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Epidemiologic Assessment of Worker Serum Perfluorooctanesulfonate (PFOS) and Perfluorooctanoate (PFOA) Concentrations and Medical Surveillance Examinations

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Perfluorooctanesulfonyl fluoride (POSF, $C_8F_{17}SO_2F$) is used to create applications for surfactants and paper, packaging, and surface (eg, carpets, textiles) protectants. Such POSF-based products or their residuals may degrade or metabolize to PFOS ($C_8F_{17}SO_3^-$). PFOS concentrates in liver and serum and results in hypolipidemia as an early effect of cumulative dosages. Male and female employees of two perfluorooctanyl-manufacturing locations (Antwerp, Belgium and Decatur, Alabama) participated in a periodic medical surveillance program that included hematology, clinical chemistry, thyroid hormone, and urinalysis testing. Serum concentrations of PFOS and perfluorooctanoate (PFOA, $C_7F_{15}CO_2^-$, used as a fluoropolymer emulsifier) were measured via mass spectrometry methods. The mean serum PFOS and PFOA concentrations for 263 Decatur employees were 1.32 parts per million (ppm; geometric mean 0.91, range 0.06–10.06 ppm) and 1.78 ppm (geometric mean 1.13, range 0.04–12.70 ppm), respectively. Mean concentrations were approximately 50% lower among 255 Antwerp workers. Adjusting for potential confounding factors, there were no substantial changes in hematological, lipid, hepatic, thyroid, or urinary parameters consistent with the known toxicological effects of PFOS or PFOA in cross-sectional or longitudinal analyses of the workers' measured serum fluorochemical concentrations. (J Occup Environ Med. *2003;45:260–270*)

Perfluorooctanesulfonyl fluoride (POSF, $C_8F_{17}SO_2F$), which is produced by an electrochemical fluorination process, is used as the basic building block to create unique chemistries through the sulfonyl fluoride moiety using conventional hydrocarbon reactions. Applications include surfactants and paper, packaging, and surface (eg, carpet, upholstery, textile) protectants. Depending upon the specific functional derivatization or the degree of polymerization, such POSF-based products or their residuals may degrade or metabolize to an undetermined degree to PFOS ($C_8F_{17}SO_3^-$). PFOS is a stable and persistent end-product that has the potential to bioaccumulate. Although not a major commercial product, PFOS has been used in some products, including firefighting foams. Another fluorochemical, the ammonium salt of perfluorooctanoate (PFOA, $C_7F_{15}CO_2^-$) is produced to be an emulsifier in the polymerization of fluoropolymers. In May 2000, the 3M Company announced that it would voluntarily cease manufacturing perfluorooctanyl-related materials after PFOS was found to be pervasive and persistent in human populations and wildlife.[1–5] Nonoccupational exposures to PFOS or precursors are not well understood at this time but could include environmental sources, consumer products, or as indirect food additives.

PFOS concentrates primarily in the liver and, to a lesser extent, in the

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plasma, of rats.[6] There appears to be significant enterohepatic circulation of PFOS with both urinary and fecal excretion.[7] Subchronic studies in rats and primates suggest that the toxicity of PFOS is dependent upon cumulative dose.[8–12] Decreased serum cholesterol was the earliest clinical chemistry response observed in primates and occurred at serum PFOS (potassium salt) concentrations above 100 parts per million (ppm).[12] Rats fed PFOS (potassium salt) at 20 ppm in the diet over a 2-year time period experienced a marginal increase in liver tumors, primarily adenomas.[13] Although the mechanism of toxicity in laboratory animals remains to be fully elucidated, PFOS toxicity may involve: 1) the inhibition of HMG CoA reductase and acyl CoA cholesterol acyl transferase activity;[14] 2) activation of the PPARα receptor;[15,16] 3) disturbances in fatty acid transport and metabolism;[17] and/or 4) altered membrane function changes and mitochondrial bioenergetics.[18,19]

There are substantial sex and species differences in elimination of PFOA with the longest serum elimination half-life reported for humans.[20–23] Besides urinary excretion, biliary excretion and reabsorption of PFOA occurs.[7] The liver is the primary target organ for PFOA-induced toxicity. PFOA produced hypolipidemia in rodents[14,24] but not in a 6-month capsule feeding study of cynomolgus monkeys.[22] Increased liver weight as a result of mitochondrial proliferation occurred in all three monkey dose groups (3, 10, and 20 mg/kg/day), although histopathologic evidence of liver injury occurred only in the highest dose group. Two-year lifetime bioassays of rats fed 300 ppm PFOA in the diet resulted in the increased incidence of tumors (adenomas) of the liver, pancreas (acinar cell), and testes (Leydig cell).[25] The hepatocellular tumors may result from a combination of oxygen stress and cell proliferation that accompanies an increase in peroxisomes. The tumors observed in the testis may be the consequence of sustained increases in estradiol as a result of aromatase induction[25–27] whereas those in the pancreas may involve the release of cholecystokinin as a result of cholestasis.[28] It remains to be determined whether these possible mechanisms are relevant to humans.

Serum measurements of PFOS and PFOA have been obtained as part of the 3M Company's effort to assure worker safety as related, in particular, to lipid and hepatic parameters because of the toxicological evidence that indicates the liver is the target organ of effect in rats and primates. The company's fluorochemical medical surveillance program is offered employees on a routine periodic basis at three 3M manufacturing facilities: Antwerp (Belgium), Cottage Grove (Minnesota), and Decatur (Alabama). Electrochemical fluorination at the Cottage Grove site involved the production of PFOA and its associated salts but not POSF.[29–31] The present study focused on the company's Antwerp and Decatur facilities, where POSF and PFOA have been produced. Although a cross-sectional assessment of the 1995 and 1997 Antwerp and Decatur medical surveillance program data was previously reported, the data were limited for several reasons: low voluntary employee participation, no inclusion of female employees, inherent limitations of the cross-sectional design, and analysis of only serum PFOS concentrations.[32] Increased employee participation in the year 2000 medical surveillance program allowed for a much larger cross-sectional analysis of both male and female employees and also enabled a 6-year longitudinal assessment of clinical chemistries in relation to PFOS, PFOA, and a calculated total organic fluorine value.

Materials and Methods

Manufacturing Sites

The manufacturing operations of the two sites are similar. Fluorochemical production occurs in several buildings. In one building, where the base product is manufactured, POSF, occurs via electrochemical fluorination. At another building, the POSF starting material is reacted to form fluorochemical amines and then further to other fluorochemicals, including alcohols and acrylates with subsequent polymerization. Synthetic fluoroelastomers are also produced at both facilities. PFOA has been routinely produced at Antwerp but only since 1999 in Decatur. However, PFOA can be a byproduct of POSF-related manufacturing at both facilities.

The fluorochemical medical surveillance program is available on a periodic voluntary basis to all Antwerp and Decatur chemical plant employees and those employees with site-wide responsibilities (eg, environmental, health and safety workers). In 2000, approximately 340 Antwerp and 500 Decatur chemical plant and site employees were eligible to participate. The surveillance program measured several serum fluorochemicals, including PFOS and PFOA as well as hematology, clinical chemistries, and thyroid hormones. Urinalyses were conducted only in Decatur.

Hematology, Clinical Chemistry, Thyroid Function, and Urinalysis

Upon collection and shipment of specimens, Allina Laboratory Services (St. Paul, MN) performed standard hematological and clinical chemistry tests for both manufacturing sites. These included the following hematological tests: hematocrit (percent), hemoglobin (gm/dL), red blood cells (RBC, 1000/mm^3), white blood cells (WBC, 1000/mm^3) and platelet count (1000/mm^3); and the following clinical chemistry tests: alkaline phosphatase (IU/L), gamma glutamyl transferase (GGT, IU/L),

aspartate aminotransferase (AST, IU/L), alanine aminotransferase (ALT, IU/L), total and direct bilirubin (mg/dL), cholesterol (mg/dL), high-density lipoprotein (HDL, mg/dL), triglycerides (mg/dL), blood glucose (mg/dL), blood urea nitrogen (BUN, mg/dL), and serum creatinine (mg/dL). Reference ranges have been relatively constant since 1994/95, although for ALT the range declined from 20–65 IU/L in 1994/95 to 1–40 IU/L in 1997 and 2000. Six thyroid tests were conducted by LabCorp (Kansas City, MO): thyroid-stimulating hormone (TSH; μIU/mL); serum thyroxine (T4; μg/dL); free thyroxine (free T4; ng/dL); serum triiodothyronine (T3; ng/dL); thyroid hormone binding ratio (THBR, previously referred to as T3 Uptake); and free thyroxine index (FTI). TSH, free T4, and T3 were determined by an immunochemiluminometric assay. T4 and THBR were determined by a cloned enzyme donor immunoassay. FTI was calculated by multiplying T4 and THBR. Urinalyses were only performed on Decatur employees via the standard urine microstick analysis, which tested for urine glucose, albumin and red blood cells.

Fluorochemical Analyses

In the 2000 fluorochemical medical surveillance program, the employees' sera samples were extracted and quantitatively analyzed for PFOS and PFOA using high-pressure liquid chromatography electrospray tandem mass spectrometry and evaluated versus an extracted curve from a human plasma matrix. All serum fluorochemical analyses were determined by Northwest Bioanalytical Laboratory Inc. (Salt Lake City, UT). Sera samples were extracted using an ion-pairing extraction procedure.[2] Evaluation of quality control samples injected during each analytical run indicated that the reported quantitative results may have varied, on average, up to 20% although most analyses were within ±10%. For all employees, serum values for PFOS and PFOA values were above the lower limit of quantitation. Results are reported in ppm.

Five other fluorochemical analytes were also analyzed in the 2000 medical surveillance program: perfluorohexanesulfonate ($C_6F_{13}SO_3^-$); N-ethyl perfluorooctanesulfonamidoacetate ($C_8F_{17}SO_2N(CH_2CH_3)CH_2COO^-$); *N*-methyl perfluorooctanesulfonamidoacetate ($C_8F_{17}SO_2N(CH_3)CH_2COO^-$); perfluorooctanesulfonamidoacetate ($C_8F_{17}SO_2NHCH_2COO^-$); and perfluorooctanesulfonamide ($C_8F_{17}SO_2NH_2$). These fluorochemicals were measured at concentrations 1 to 3 orders of magnitude lower than PFOS and PFOA and are therefore not presented. A total organic fluorine (TOF) value was determined by calculating the percent of each of the seven specific fluorochemical's molecular weight that was attributed to organic fluorine (eg, PFOS, 64.7%; PFOA, 69.0%) multiplied by the ppm measured for each fluorochemical and then summed across all seven fluorochemicals.

Serum fluorochemical analyses were conducted at different laboratories in the previous surveillance years (1994 [Decatur], 1995 [Antwerp], and 1997 [both facilities]) using slight variations of the Hansen et al. method.[2] In those years, only PFOS and PFOA were quantified. Therefore, TOF for the longitudinal assessment was based only on PFOS and PFOA.

Data Analyses

For the cross-sectional analyses, associations between PFOS, PFOA, or TOF and each hematological and clinical chemistry test and thyroid hormone assay were evaluated with descriptive statistics, analysis of variance, and multivariable regression. For stratified analyses, employees were divided into quartiles of their serum PFOS distribution. Age, body mass index, current alcohol consumption (drinks per day) and cigarette use (cigarettes smoked per day), years worked at Antwerp or Decatur, and type of job (production versus nonproduction) were potential confounding factors that were considered in the analyses. Production jobs included cell operators, chemical operators, mill operators, and crew supervisors. Nonproduction jobs included engineers, quality-assurance laboratory and research workers, and administration (eg, managers, clerical staff).

Logistic regression was used to calculate adjusted odds ratios for the quartile distribution of employees who had liver function tests above the laboratory's reference ranges. In addition, the individual continuous dependent variables (lipids, hepatic enzymes or thyroid hormones) were fitted in multivariable regression analyses with PFOS, PFOA, or TOF analyzed as an independent continuous variable(s). The statistical significance of the fluorochemical coefficient was considered at $P < 0.05$. Natural log transformations of the dependent variables were performed when necessary to normalize variables and to enhance model fit. Study results were analyzed using the SAS System (SAS Institute, Cary, NC).[33,34]

For the longitudinal assessment, lipid and hepatic clinical chemistry tests were evaluated as repeated measures incorporating the random subject effect fitted to a mixed model by the MIXED procedure in the SAS statistical package.[35] Restricted maximum likelihood estimates of variance parameters were computed. Adjusted regression models were built by introducing all covariates (see below) and testing the covariance structure. Equal spacing was assumed given there were approximately 3 years between each medical surveillance examinations. Based on goodness-of-fit tests, the autoregressive was routinely considered the best covariance structure for the mixed models. Variables included PFOS (or PFOA or TOF), years of observation (ie, follow-up), the interaction term of PFOS and years of observation, age, body mass index

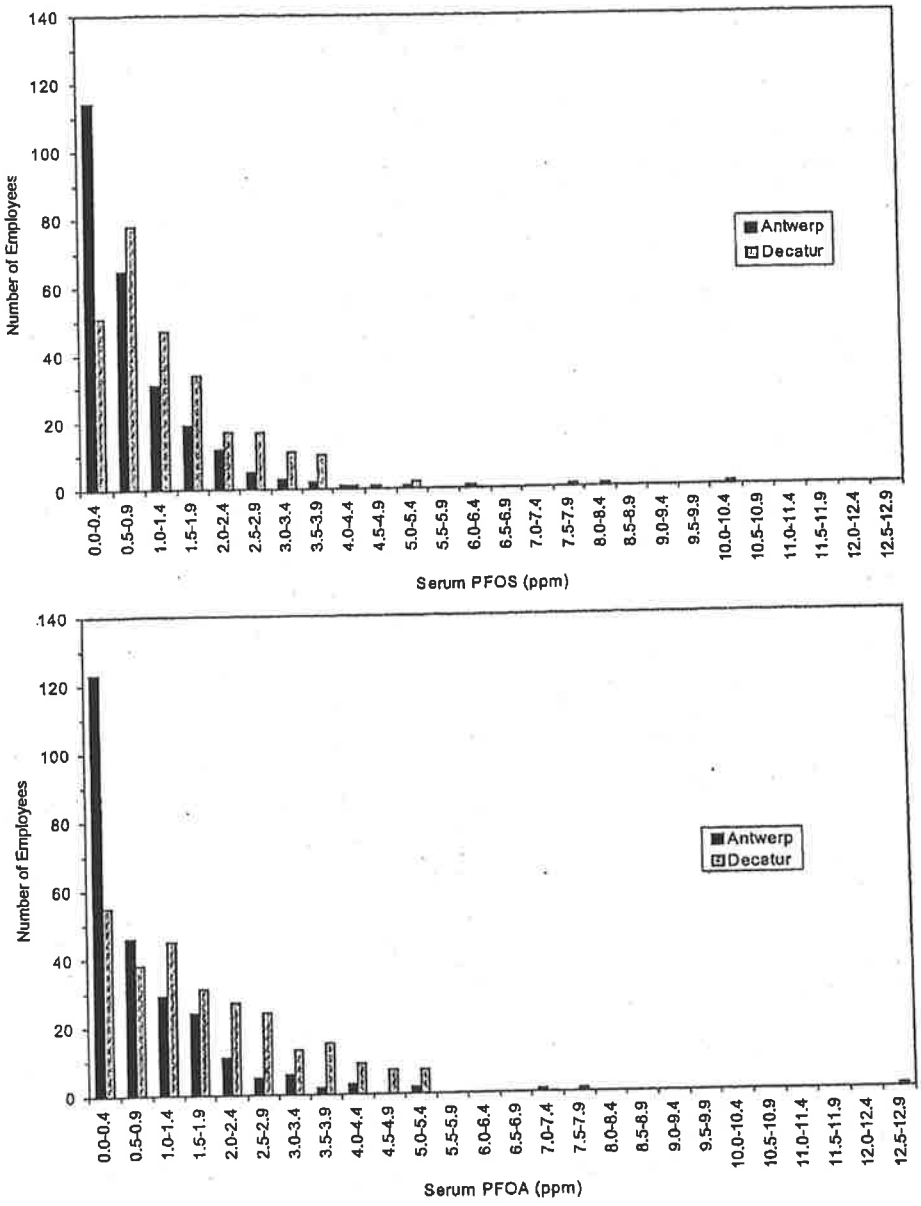

Fig. 1. Distribution of Antwerp and Decatur employees by their serum PFOS and PFOA concentrations.

(BMI), cigarettes smoked per day, alcohol drinks per day, location, year at first entry, and baseline years worked. Serum triglycerides was also considered a potential confounding factor for all hepatic clinical chemistry analyses (cross sectional and longitudinal).

Results

Cross-sectional Analysis

Altogether, there were 255 (75%) Antwerp employees (206 male and 49 female) and 263 (52%) Decatur employees (215 male, 48 female) who voluntarily participated in the 2000 fluorochemical medical surveillance program. Seventy-three percent of the Antwerp male employee participants and 75% of the Decatur employee participants worked in production activities. Only 12% of the Antwerp female employees worked in production activities compared with 63% of the Decatur female employees.

Presented in Fig. 1 are the distributions of serum PFOS and PFOA concentrations among the Antwerp and Decatur employee participants. The arithmetic mean serum PFOS concentration among all Antwerp subjects was 0.80 ppm (range 0.04–6.24 ppm) with a geometric mean of 0.44 ppm (95% CI = 0.38–0.51). For PFOA, the arithmetic mean was 0.84 ppm (range 0.01–7.04 ppm) with a geometric mean of 0.33 ppm (95% CI = 0.27–0.40). The arithmetic mean serum PFOS concentration among all Decatur participants

TABLE 1
Mean and Standard Deviation Comparisons Between Antwerp and Decatur Employees' Serum PFOS, PFOA, TOF, Demographic and Clinical Chemistry Results, by Gender

	Males				Females			
	Antwerp (N = 206)		Decatur (N = 215)		Antwerp (N = 49)		Decatur (N = 48)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PFOS	0.96[d]	0.97	1.40	1.15	0.13[d]	0.10	0.93	0.81
PFOA	1.03[d]	1.09	1.90	1.59	0.07[d]	0.17	1.23	1.18
TOF	1.60[d]	1.34	2.65	2.00	0.17[d]	0.20	1.76	1.50
Age	37[d]	9	43	9	36	7	42	9
BMI	24.8[d]	3.0	28.8	4.4	22.8[d]	3.2	27.7	5.9
Years worked	13[c]	8	16	13	12[a]	7	13	10
Alcoholic drinks/day	1.1[d]	1.1	0.1	0.3	0.5[d]	0.4	0.1	0.1
Cholesterol	218	41	215	42	208	36	200	39
HDL	55[d]	15	44	10	68[a]	16	59	12
Triglycerides	124[d]	87	191	124	94[d]	45	133	152
Alk Phos	60[d]	15	74	20	46[a]	13	65	18
GGT	23[d]	17	31	18	12[d]	7	18	15
AST	23[c]	6	26	8	18	6	20	7
ALT	23[d]	10	35	16	13[d]	6	19	10
Total Bilirubin	1.0[d]	0.3	0.7	0.2	0.8[b]	0.3	0.6	0.2

[a] $p < .05$ compared to Decatur (student t test).
[b] $p < .01$ compared to Decatur (student t test).
[c] $p < .001$ compared to Decatur (student t test).
[d] $p < .0001$ compared to Decatur (student t test).

was 1.32 ppm (range 0.06 to 10.06 ppm) with a geometric mean of 0.91 ppm (95% CI = 0.82–1.02). For PFOA, the arithmetic mean was 1.78 ppm (range 0.04–12.70 ppm) with a geometric mean of 1.13 ppm (95% CI = 0.99–1.30).

Antwerp male employees compared with their Decatur counterparts had lower mean serum PFOS and PFOA concentrations; were younger; had lower BMIs; drank more alcoholic beverages per day; and had higher mean HDL and total bilirubin and lower triglyceride and hepatic clinical chemistry values (Table 1). Similar findings were observed between Antwerp and Decatur female employees (Table 1).

Presented in Table 2 are the mean, standard deviation, and range of demographic, clinical chemistry, and thyroid hormone results stratified by the serum PFOS quartile distribution for the combined 421 Antwerp and Decatur production and nonproduction male employees. (Note: The median values [data not shown in Table 2] by PFOS quartile, for PFOS, PFOA, and TOF were, respectively: quartile 1: [0.29, 0.25, 0.43 ppm]; quartile 2 [0.59, 0.86, 1.14 ppm]; quartile 3 [1.17, 1.20,1.88 ppm]; and quartile 4 [2.46, 2.43, 4.06 ppm].) As noted in the footnote to Table 2, the demographic and clinical values reflect the higher percentage of Antwerp employees in the lowest PFOS quartile and the higher percentage of Decatur employees in the upper PFOS quartiles. The fourth quartile had statistically significant higher mean values than the first quartile for triglycerides, alkaline phosphatase, ALT and T3. The fourth quartile had significantly lower mean values for drinks per day and total bilirubin compared to the first quartile. There were no significant mean differences between quartiles for cigarettes smoked, hematology, blood glucose, BUN, serum creatinine or urinalyses (data not shown).

Similar to Table 2, presented in Table 3 are the serum PFOS quartile distributions for Antwerp and Decatur production and nonproduction female employees. Again, the Antwerp employees predominated in the lowest serum PFOS quartile and Decatur female employees predominated in the highest quartile. [Note: The median values [data not shown], by quartile, for PFOS, PFOA and TOF were, respectively: quartile 1: [0.08, 0.02, 0.09 ppm]; quartile 2 [0.13, 0.05, 0.14 ppm]; quartile 3 [0.37, 0.36, 0.59 ppm]; and quartile 4 [1.34, 1.39, 2.66 ppm].) The fourth quartile had significantly higher mean values than the first quartile for age, BMI, alkaline phosphatase, and GGT. The fourth quartile had significantly lower mean values for alcoholic drinks per day and total bilirubin. There were no significant mean differences for cigarettes smoked, hematology, blood glucose, BUN, serum creatinine, or urinalyses (data not shown).

Summarized in Table 4 are the number of Antwerp and Decatur employees (and percentages) who had hepatic enzyme tests above reference range values, stratified by the quartiles of the serum PFOS distribution. Among male employees, 12% of the employees in the fourth quartile had above reference range values for ALT and GGT compared with 4 to 8% in the first through third quartiles. For the total liver panel, 23% of

TABLE 2
Antwerp and Decatur Production and Non-Production* (N = 421) Male Employees' PFOS, PFOA, TOF, Demographic, Clinical Chemistry and Thyroid Hormone Results by Quartile of Serum PFOS Distribution*

	Quartile 1 (N = 105)			Quartile 2 (N = 105)			Quartile 3 (N = 106)			Quartile 4 (N = 105)		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
PFOS	0.27[2,3,4]	0.11	0.04–0.42	0.60[1,3,4]	0.12	0.43–0.81	1.19[1,2,4]	0.24	0.82–1.68	2.69[1,2,3]	1.09	1.69–10.06
PFOA	0.54[2,3,4]	0.77	0.01–4.03	1.21[1,4]	1.19	0.06–7.04	1.45[1,4]	1.10	0.12–7.48	2.70[1,2,3]	1.63	0.25–12.70
TOF	0.62[2,3,4]	0.58	0.05–3.03	1.40[1,3,4]	0.89	0.38–5.69	2.12[1,2,4]	0.87	0.98–6.61	4.41[1,2,3]	1.72	1.92–12.23
Age	38[3]	10	23–60	41	10	21–63	42[1]	9	22–61	40	9	27–60
BMI	25.8	4.0	19.2–40.8	26.9	4.0	19.0–37.3	27.3	4.5	17.2–50.1	27.2	4.5	17.8–45.5
Years worked	12[3]	10	1–38	15	12	2–38	16[1]	11	1–38	15	10	2–38
Drinks/day	0.9[3,4]	1.0	0–5	0.6	0.9	0–4	0.5[1]	0.9	0–6	0.5[1]	0.9	0–5
Cholesterol	214	41	140–331	214	43	121–308	215	39	105–303	222	44	122–384
HDL	54	15	31–121	47	11	29–80	48	13	24–100	48	15	26–119
Triglycerides	131[4]	95	32–527	155	102	35–633	169	123	32–731	177[1]	123	39–796
Alk Phos	61[3,4]	16	26–98	67	18	30–142	69[1]	21	30–160	70[1]	19	21–126
GGT	24	16	7–111	29	22	7–144	26	15	6–89	30	17	7–85
AST	25	8	13–58	25	6	16–49	24	7	7–51	25	9	13–69
ALT	26[4]	13	10–91	28	11	10–63	28	14	6–103	33[1]	19	8–99
Total Bilirubin	1.0[3,4]	0.3	0.5–2.0	0.9	0.3	0.3–2.0	0.8[1]	0.3	0.4–2.0	0.8[1]	0.3	0.4–2.2
TSH	2.0	1.2	0.03–5.7	3.1	6.6	0.5–65.3	2.1	2.0	0.2–18.8	2.5	2.8	0.5–21.5
T4	8.3	1.4	0.5–11.5	8.2	1.4	4.2–12.0	8.3	1.5	3.3–12.9	8.4	1.4	4.7–11.4
Free T4	1.1	0.2	0.9–1.5	1.1	0.1	0.6–1.4	1.1	0.2	0.4–1.6	1.1	0.2	0.8–1.6
T3	124[4]	17	94–164	128	20	86–186	127	21	91–196	132[1]	22	87–190

* Number of male employees by location, production (P) and non-production (NP) category and quartile (percent in parenthesis).

	Q1		Q2		Q3		Q4	
	P	NP	P	NP	P	NP	P	NP
Antwerp	38	38	38	12	38	4	36	2
Decatur	7	22	40	15	51	13	63	4
Total	45 (43)	60 (57)	78 (74)	27 (26)	89 (84)	27 (16)	99 (94)	6 (6)

[1] Mean is significantly different ($P < .05$, Bonferroni (Dunn) t test) from the mean of the 1st quartile; [2] 2nd quartile; [3] 3rd quartile; [4] 4th quartile.

the male employees had one or more liver clinical chemistry tests above the reference range value compared with 14 to 16% of the male employees in the lower three quartiles. Odds ratios were calculated for each quartile for those employees above or below reference ranges as listed in Table 4 (quartile one reference odds ratio = 1.0). The odds ratios were adjusted for the potential confounding effects of age, BMI, alcohol, cigarettes, and location. For ALT, the odds ratios for the second, third, and fourth quartiles were (95% CI in parentheses) 0.6 (95% CI = 0.1–2.8), 1.2 (0.3–4.8), and 2.1 (0.6–7.3), respectively. For GGT, the odds ratios were 1.3 (0.4–4.1), 0.9 (0.3–3.1), and 2.0 (0.7–5.8), respectively. For the total liver panel, the odds ratios were: 1.1 (0.5 to 2.3), 1.1 (0.4–2.3), and 1.6 (0.7–3.3), respectively. None of the odds ratios were statistically significant ($P < 0.05$). Logistic models did not meet satisfactory convergence criteria because of the few male subjects with values above the reference ranges for alkaline phosphatase or AST or for female employees for any liver function analysis in Table 4.

Using multivariable regression analysis and adjusting for potential confounders, serum PFOS was positively associated with the natural log of serum cholesterol (PFOS coefficient = 0.020, P value = 0.04) and triglycerides (PFOS coefficient = 0.066, P value = 0.01), although these associations contributed minimally (partial $R^2 < 0.01$ and 0.03, respectively) to the variation explained in the models (cholesterol model adjusted $R^2 = 0.06$; triglyceride model adjusted $R^2 = 0.27$). PFOA and TOF were also positively associated with cholesterol and triglycerides with similar variations explained. These modest positive associations for PFOS or PFOA, however, were opposite the expected direction based on the known toxicity of these compounds. HDL was not significantly associated with PFOS or PFOA. Adjusting for their potential confounders, the hepatic enzyme and bilirubin analyses were not significantly associated with PFOS or PFOA. Multivariable regression analyses of the thyroid hormones resulted in no significant associations with PFOS or PFOA except for a positive association with the natural log of T3 (PFOS coefficient = 0.015, P value = 0.04; PFOA coefficient = 0.016, P value = 0.01), which, again, contributed minimally to the variation explained in the model (partial $R^2 = 0.01$).

Longitudinal Analysis

A total of 174 Antwerp and Decatur male employees participated in

TABLE 3
Antwerp and Decatur Production and Non-Production* (n = 97) Female Employees' PFOS, PFOA, TOF, Demographic, Clinical Chemistry and Thyroid Hormone Results by Quartile of Serum PFOS Distribution*

	Quartile 1 (n = 24)			Quartile 2 (n = 24)			Quartile 3 (n = 25)			Quartile 4 (n = 24)		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
PFOS	0.07[3,4]	0.02	0.04–0.10	0.13[4]	0.03	0.10–0.19	0.39[1]	0.15	0.20–0.70	1.51[1,2,3]	0.76	0.77–3.62
PFOA	0.04[3,4]	0.04	0.01–0.23	0.07[4]	0.07	0.02–0.34	0.61[1]	0.74	0.04–3.50	1.88[1,2,3]	1.20	0.25–5.41
TOF	0.09[3,4]	0.04	0.05–0.26	0.17[3,4]	0.07	0.09–0.35	0.80[1,2,4]	0.61	0.21–3.02	2.77[1,2,3]	1.44	0.86–7.81
Age	34[4]	9	24–52	37[4]	7	25–52	39	9	25–58	44[1,2]	6	30–52
BMI	22.8[4]	2.7	18.4–28.3	23.9[4]	4.3	17.3–32.3	25.5	6.1	18.3–45.3	28.7[1,2]	5.7	20.3–41.5
Years Worked	11	8	1–29	15	7	3–29	12	9	2–27	14	10	3–32
Drinks/day	0.4[4]	0.4	0–1	0.4[4]	0.4	0–1	0.3	0.4	0–2	0[1,2]	0.1	0–1
Cholesterol	207	39	132–274	203	39	138–302	200	32	139–271	208	42	129–313
HDL	66	16	46–121	65	16	33–104	63	15	38–104	60	13	36–91
Triglycerides	93	48	26–248	91	41	24–172	107	53	32–233	164	206	42–1049
Alk Phos	50[4]	16	22–81	44[3,4]	11	20–65	59[2]	16	32–91	69[1,2]	18	41–100
GGT	11[4]	7	2–32	13	8	5–41	14	6	7–30	22[1]	21	6–97
AST	19	5	11–31	18	7	9–43	19	5	11–33	19	7	7–39
ALT	13	5	8–35	16	11	6–58	16	6	7–36	19	10	6–47
Total Billrubin	0.8[3,4]	0.2	0.5–1.2	0.8[3,4]	0.3	0.2–1.7	0.6[1,2]	0.2	0.3–1.0	0.5[1,2]	0.1	0.3–0.8
TSH	2.2	1.2	0.03–4.9	2.2	1.5	0.03–6.7	2.5	1.4	0.7–6.5	2.3	1.0	1.0–5.2
T4	10.2	2.0	6.6–13.8	9.8	3.1	4.6–18.3	9.9	2.3	5.8–15.1	9.1	2.1	5.8–14.2
Free T4	1.1	0.1	0.8–1.3	1.2	0.7	0.7–4.6	1.1	0.1	0.9–1.3	1.0	0.1	0.7–1.2
T3	145	28	98–191	147	53	81–345	133	31	86–228	127	28	86–196

* Number of female employees by location, production (P) and non-production (NP) category and quartile (percent in parenthesis).

	Q1		Q2		Q3		Q4	
	P	NP	P	NP	P	NP	P	NP
Antwerp	3	20	2	17	1	6	0	0
Decatur	0	1	1	4	7	11	22	2
Total	3 (12)	21 (88)	3 (12)	21 (88)	8 (32)	17 (68)	22 (92)	2 (8)

[1] Mean is significantly different ($P < .05$, Bonferroni (Dunn) t test) from the mean of the 1st quartile; [2]2nd quartile; [3]3rd quartile; [4]4th quartile.

the year 2000 medical surveillance program and at least one of the two previous program years: 64 (37%) participated in both 1994/95 and 2000 (Antwerp = 45, Decatur = 19); 69 (39%) of the longitudinal group participated in both 1997 and 2000 (Antwerp = 34, Decatur = 35); and 41 (24%) participated in all 3 years (Antwerp = 21, Decatur = 20). For purposes of brevity, these three subpopulations are referred to as subgroups A, B, and C, respectively.

Presented in Table 5 are the mean PFOS, PFOA, and TOF concentrations for these three subgroups by manufacturing site and year of the medical surveillance program. Serum PFOS declined over the 6-year time period whereas mean serum PFOA concentrations increased for subgroups B and C.

Serum PFOS was not a significant predictor of cholesterol or triglycerides in the longitudinal analyses (Table 6). PFOA and TOF, however, were positively associated with cholesterol as well as triglycerides. This association was primarily attributed to the 21 Antwerp employees represented in subgroup C whose mean serum PFOA levels increased over the 6-year period from 1.32 ppm to 2.06 ppm whereas their serum PFOS levels declined from 2.10 ppm to 1.53 ppm. During the same time period, their mean cholesterol values increased from 208 mg/dL to 229 mg/dL and their triglyceride levels increased from 85 mg/dL to 123 mg/dL. Their BMIs increased from 23.4 to 24.3. There were no significant PFOS, PFOA, or TOF coefficients associated with changes in HDL or the various liver function tests (data not shown) adjusting for the potential confounders.

Discussion

Medical surveillance programs in the workplace, including the present fluorochemical program, are usually voluntary. High participation rates in these voluntary programs are important to identify employees with abnormal test results, minimize participation bias, and increase the statistical power to detect subtle abnormalities.[36] Also, nonparticipation may not allow for an adequate characterization of the distribution of chemical-specific serum concentrations in employee biomonitoring analyses. We previously addressed this latter question by conducting a random sample biomonitoring study of Decatur employees and 80% of those eligible participated.[37] The serum distributions of PFOS and PFOA concentrations were comparable to previous data collected

TABLE 4
Number of Participants (Percent in Parenthesis) by Employee Population Who Had Above Reference Range Values for Hepatic Clinical Chemistry Tests by Quartile of Serum PFOS Distribution

	Alkaline Phosphatase				AST				ALT				GGT				Total Liver Panel*			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Antwerp & Decatur																				
Male Employees Production and[1] Non-Production	0 (0)	1 (1)	3 (3)	2 (2)	3 (3)	1 (1)	1 (1)	4 (4)	4 (4)	4 (4)	7 (7)	13 (12)	6 (6)	8 (8)	6 (6)	12 (12)	15 (14)	17 (16)	17 (16)	24 (23)
Female Employees Production and[2] Non-Production	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (8)	0 (0)	2 (8)	0 (0)	2 (8)

* Includes Alkaline Phosphatase, AST, ALT, GGT, Total and Direct Bilirubin (at least one of these tests were above upper reference range).
[1] See Table 2 PFOS quartile distribution.
[2] See Table 3 PFOS quartile distribution.

TABLE 5
Mean and Standard Deviation of PFOS, PFOA, TOF for Three Employee Subgroups Who Participated in Fluorochemical Medical Surveillance Program Between 1994 and 2000

	Subgroup A (N = 64)				Subgroup B (N = 69)				Subgroup C (N = 41)					
	1994/1995		2000		1997		2000		1994/1995		1997		2000	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PFOS	2.13	2.16	1.36	1.24	1.36	1.37	1.20	0.91	2.13	1.46	2.09	1.54	1.65	1.62
PFOA	1.36	1.63	1.59	1.72	1.22	0.97	1.49[a]	1.05	1.41	1.09	1.90[a]	1.87	1.77[a]	1.22
TOF	2.32	2.09	1.98	1.69	1.73	1.47	1.80	1.16	2.35	1.36	2.66[a]	1.72	2.29	1.60

[a]comparison between 1994/1995 and 2000 mean data, $P < .05$

through past, as well as the present fluorochemical medical surveillance programs; thus, we have concluded that this voluntary fluorochemical medical surveillance program has likely presented a nonbiased assessment of the employees' serum fluorochemical distribution.

The company's 2000 fluorochemical medical surveillance program had three times the number of participants compared to previous surveillance years. The voluntary participation rates for the present assessment were 53% (Decatur) and 75% (Antwerp) and were higher than those in previous surveillance years. This increase in employee participation was likely the consequence of at least two factors: 1) heightened employee awareness about their serum fluorochemical values; and 2) the company's announcement that it would voluntarily cease production of perfluorooctanyl chemistry in certain repellents and surfactants because of the pervasive and persistent nature of PFOS.

We observed several demographic and lifestyle differences between the Antwerp and Decatur employees. In particular, Antwerp male employees were younger, had lower BMIs, and drank more alcoholic beverages than their Decatur counterparts. These differences can be important confounding variables when analyzing lipid and hepatic clinical chemistries.

In the cross-sectional analysis, the positive association in the multivariable models between PFOS and serum cholesterol is contrary to the substantial body of toxicological literature that suggests a negative association in laboratory animals.[12,13,18,38] In a 6-month primate PFOS capsule study, triglycerides were unaffected but decreased serum cholesterol was an early toxicological response that occurred at serum PFOS levels above 100 ppm.[12] This serum PFOS concentration is approximately ten times greater than the highest employee value (10.06 ppm) measured in the present study. Accordingly, the positive association we observed between measured serum PFOS concentrations and total cholesterol appears spurious.

The serum PFOA concentrations measured in these 518 employees were lower than those measured among the company's manufacturing employees at its Cottage Grove facility (the primary site of PFOA manufacture) whose serum PFOA levels have been assayed as high as 114 ppm (mean = 5 ppm; median = 1 ppm).[31] Mean serum trigylceride levels were greater among the Cottage Grove APFO production workers with the highest (≥10 ppm) PFOA serum concentrations although adjustment for potential confounders have provided inconclusive

TABLE 6
Longitudinal Analyses of Serum Cholesterol or Triglycerides Levels by PFOS, PFOA or TOF and Other Covariates for 174 Male Employees

	Cholesterol#		Triglycerides#	
	Coefficient	95% C.I.	Coefficient	95% C.I.
PFOS Model*				
PFOS	0.010	(−0.005)–0.025	0.025	(−0.015)–0.065
Years Observed	0.0009	(−0.008)–0.010	−0.004	(−0.029)–0.021
PFOS × Years Obs	−0.0004	(−0.004)–0.003	0.006	(−0.004)–0.015
PFOA Model*				
PFOA	0.032	0.013–0.051	0.094	0.045–0.144
Years Observed	0.005	(−0.004)–0.014	0.007	(−0.017)–0.031
PFOA × Years Obs	−0.005	(−0.001)–(−0.002)	−0.008	(−0.018)–0.002
TOF Model*				
TOF	0.021	0.006–0.035	0.053	0.014–0.093
Years Observed	0.004	(−0.005)–0.014	−0.0005	(−0.027)–0.026
TOF × Years Obs	−0.003	(−0.005)–0.0003	−0.0005	(−0.008)–0.007

Natural log
* Adjusted for age, BMI, drinks/day, cigarettes/day, location, entry period and baseline years worked.

results.[31] The positive associations observed between PFOA and triglycerides in the present study are inconsistent with the known hypolipidemic effect of this compound in rats that is thought to be associated with activation of the nuclear receptor, peroxisome proliferator-activated receptor (PPARα).[14,16,39] Haughom and Spydevold have suggested that this hypolipidemic effect results from an overall decrease in lipoprotein formation due to decreased activities of hydroxymethylglutaryl CoA reductase and acyl-CoA:cholesterol acyltransferase as well as a reduction in fatty acid synthesis.[14] The relevance of the PPARα-mediated response in humans has been debated because of the greatly decreased expression of PPARα in humans and other nonresponsive species.[40,41] On the other hand, PPARγ, a nuclear receptor expressed mainly in adipose tissue, has been shown to be activated by the antidiabetic thiazolidinendiones, which upregulates glycerol kinase activity stimulating increased hepatic triglycerides.[42] Whether this mode of action is plausible for PFOA in humans is not known. Mouse and human PPARγ$_1$ were unresponsive to PFOA when tested at a range of 0.5 to 40 μM in a cell transfection assay.[43] In a 6-month oral toxicity study of PFOA (ammonium salt) in male cynomolgus monkeys, PFOA was significantly associated with triglycerides in the high-dose group.[22] This association was observed in measurements taken after one month of dosing at which time group mean triglyceride was significantly higher than control values as well as within group pretreatment values. At the end of the study mean triglyceride was elevated compared to time related controls but not to the animals' pretreatment values. However, only two primates were evaluated in the high-dose group at the end of study. Inspection of individual values for PFOA serum concentration and serum triglyceride values did not reveal a meaningful association between these two parameters (John Butenhoff, personal communication). Therefore, although the possibility cannot be discounted that PFOA may activate the PPARγ nuclear receptor with a resulting increase in triglycerides, the evidence for this mechanism remains equivocal.

Adjusting for potential confounding factors, there were no substantial associations between hepatic enzymes and the employees' serum PFOS concentrations. This observation is consistent with results from the 6-month PFOS capsule feeding study where no overt hepatic toxicity was observed in the two lower dose groups (0.03 or 0.15 mg/kg/day) whose mean serum concentrations measured 16 and 83 ppm (males) and 13 and 67 ppm (females), respectively.[12] The 0.75 mg/kg/day treatment group did show hepatocellular hypertrophy and lipid vacuolation along with other signs of toxicity and two of six male monkeys died (no female deaths). No significantly increased differences from control or pretreatment values were observed at end-of-study for serum levels of alkaline phosphatase or ALT in the high dose group. Mean serum concentrations for male and female monkeys at this dose was 170 ppm. The liver:serum concentration ratio in the primate was approximately 1:1 to 2:1 for all treatment groups.

We observed a positive association between PFOS and T3 in the longitudinal assessment. There is unlikely any clinical relevance for this association. No other thyroid hormone was associated with PFOS and the observation is contrary to the primate study, which showed lowered T3 values without an indication of a hypothyroid compensatory increase in TSH, hypolipidemia or thyroid gland histological changes.

Retrospective cohort mortality studies have not reported statistically

significant standardized mortality ratios for all cancer or liver cancer deaths at either the Decatur or Cottage Grove manufacturing sites.[44–46] The Decatur mortality study did observe three deaths from bladder cancer compared to 0.2 expected (standarized mortality ratio = 12.8; 95% CI = 2.6–37.4) in the subgroup of workers with the highest potential exposure to perfluorooctanesulfonyl fluoride (POSF)-based materials.[44] Alexander et al. did not determine whether this association was fluorochemical-related or possibly due to other nonfluorochemical occupational exposures, nonoccupational exposures or chance. PFOS did not result in any bladder tumors in the high dose group (20 ppm) of a 2-year bioassay of rats.[13] The present study found no differences among Decatur employees' serum PFOS concentrations and their urinalyses. Additional epidemiologic research is ongoing to further understand the bladder cancer mortality association among the Decatur workforce.

Although we were able to perform a longitudinal assessment of the medical surveillance data, several limitations remained in our analyses. Only 41 of the 175 employees in the longitudinal analysis participated in all three surveillance years. Insufficient numbers prevented longitudinal analyses of the female employees. Because 3M announced a phase-out of the production of perfluorooctanyl chemistry-related materials, it is doubtful whether there will be more employees who can be included in future longitudinal assessments. Given the variability inherent in the analytical method and the different laboratories used, serum measurements of PFOS and PFOA may have varied ±20% although most were ±10%. This experimental error may have masked associations with lipid or hepatic clinical chemistries although the range of PFOS and PFOA measured was relatively consistent throughout the study time period. Our findings suggest that employees' measured serum PFOS concentrations have either remained constant or declined slightly during this 6-year time period. However, serum PFOA levels trended slightly upwards during the study period, which may have been the result of increased production.

In summary, a cross-sectional analysis of 421 male and 97 female POSF and PFOA production and nonproduction employees, as well as a longitudinal analysis over a 6-year time period of 174 male employees, have not shown substantial changes in lipid or hepatic clinical chemistry test results that are consistent with the known toxicological effects of these compounds. This finding was not unexpected because these employees' average serum concentrations were considerably lower than those known to cause the earliest clinical effects in laboratory animals.

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Perfluorooctanesulfonate and Other Fluorochemicals in the Serum of American Red Cross Adult Blood Donors

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Perfluorooctanesulfonyl fluoride-based products have included surfactants, paper and packaging treatments, and surface protectants (e.g., for carpet, upholstery, textile). Depending on the specific functional derivatization or degree of polymerization, such products may degrade or metabolize, to an undetermined degree, to perfluorooctanesulfonate (PFOS), a stable and persistent end product that has the potential to bioaccumulate. In this investigation, a total of 645 adult donor serum samples from six American Red Cross blood collection centers were analyzed for PFOS and six other fluorochemicals using HPLC-electrospray tandem mass spectrometry. PFOS concentrations ranged from the lower limit of quantitation of 4.1 ppb to 1656.0 ppb with a geometric mean of 34.9 ppb [95% confidence interval (CI), 33.3–36.5]. The geometric mean was higher among males (37.8 ppb; 95% CI, 35.5–40.3) than among females (31.3 ppb; 95% CI, 30.0–34.3). No substantial difference was observed with age. The estimate of the 95% tolerance limit of PFOS was 88.5 ppb (upper limit of 95% CI, 100.0 ppb). The measures of central tendency for the other fluorochemicals (*N*-ethyl perfluorooctanesulfonamidoacetate, *N*-methyl perfluorooctanesulfonamidoacetate, perfluorooctanesulfonamidoacetate, perfluorooctanesulfonamide, perfluorooctanoate, and perfluorohexanesulfonate) were approximately an order of magnitude lower than PFOS. Because serum PFOS concentrations correlate with cumulative human exposure, this information can be useful for risk characterization.
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In May 2000, the 3M Company (3M) announced that it would voluntarily cease manufacturing materials based on perfluorooctanesulfonyl fluoride (POSF; $C_8F_{17}SO_2F$) after a metabolite of this compound, perfluorooctanesulfonate (PFOS; $C_8F_{17}SO_3^-$), was found to be widespread in human populations and wildlife (3M Company 2003; Giesy and Kannan 2001; Hansen et al. 2001; Kannan et al. 2001a, 2001b, 2002a, 2002b). Using POSF as a basic building block, unique chemicals were created by further reactions with functionalized hydrocarbon molecules. Major applications of these POSF-based products have included surfactants, paper and packaging treatments, and surface protectants (e.g., for carpet, upholstery, textile). Depending on the specific functional derivatization or the degree of polymerization, such POSF-based products may degrade or metabolize, to an undetermined degree, to PFOS, a stable and persistent end product that has the potential to bioaccumulate. Although not a major commercial product, PFOS has been used in some products, including fire-fighting foams.

The mechanisms and pathways leading to the presence of PFOS in human blood are not well characterized but likely involve environmental exposure to PFOS or to precursor molecules and residual levels of PFOS or PFOS precursors in industrial and commercial products. PFOS has been detected at low parts per billion (nanogram per milliliter) concentrations in the general population (3M Company 2003; Hansen et al. 2001; Olsen et al. 2003b), although the scope and sample size of these investigations have been limited. Serum PFOS concentrations among production employees working in POSF-related processes have averaged between 0.5 and 2 ppm depending on work activity (range < 0.1–12 ppm) (Olsen et al. 1999, 2003a, 2003c). A large body of toxicology and epidemiology data is available for review regarding PFOS [3M Company 2003; Organisation for Economic Co-operation and Development (OECD) 2002]. Results from several repeat-dose toxicologic studies consistently demonstrated that the liver is the primary target organ (3M Company 2003; OECD 2002). Liver cell hypertrophy and reduction in serum cholesterol are early responses to PFOS that occur in rats as well as monkeys. Both species display an apparent threshold for the toxic effects of PFOS that can be expressed in terms of cumulative dose or body burden, with no observable response at lower cumulative doses or body burdens. Following the company's announcement to voluntarily cease production, the U.S. Environmental Protection Agency (EPA) finalized a significant new use rule to regulate PFOS and related chemicals (U.S. EPA 2002).

The purpose of this study was to better characterize the distribution of PFOS and six other fluorochemicals, some of which are PFOS precursors, in a large adult population by analyzing serum samples obtained from donors at six American Red Cross blood banks. An assessment of the serum fluorochemical distribution was performed in relation to three demographic attributes (age, sex, and location) of the anonymous blood donors.

Materials and Methods

Fluorochemicals

The seven analytes detected and quantified in this study were PFOS, *N*-ethyl perfluorooctanesulfonamidoacetate [PFOSAA; $C_8F_{17}SO_2N(CH_2CH_3)CH_2COO^-$], *N*-methyl perfluorooctanesulfonamidoacetate [M570; $C_8F_{17}SO_2N(CH_3)CH_2COO^-$], perfluorooctanesulfonamidoacetate (M556; $C_8F_{17}SO_2NHCH_2COO^-$), perfluorooctanesulfonamide (PFOSA; $C_8F_{17}SO_2NH_2$), perfluorooctanoate (PFOA; $C_7F_{15}COO^-$), and perfluorohexanesulfonate (PFHS; $C_6F_{13}SO_3^-$).

PFOSAA is an oxidation product of *N*-ethyl perfluorooctanesulfonamidoethanol (N-EtFOSE), which was primarily used in paper and packaging protectant applications. M570 is an oxidation product of *N*-methyl

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The laboratory analysis of the seven fluorochemicals was provided by a dedicated team at Tandem Labs (formerly Northwest Bioanalytical; Salt Lake City, UT, USA). Individuals included A. Hoffman, C. Sakashita, P. Bennett, R. Foltz, S. Newman, T. Peacock, and E. Yardimici. We also thank J. Butenhoff and K. Young for their contributions.

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perfluorooctanesulfonamidoethanol, which was used primarily in surface treatment applications (e.g., carpets, textiles). Therefore, PFOSAA and M570 can be considered markers of consumer-related exposure. Both PFOSAA and M570 can metabolize to M556 and PFOSA, which in turn can later metabolize to PFOS. Unlike PFOSAA and M570, other POSF-related analytes (M556, PFOSA, and PFOS) are not specific to any one consumer application. PFOA and PFHS are not precursors or metabolites of PFOS. Ninety-seven percent of PFOA that was produced by 3M was used by its industrial customers and in its own processes as a fluoropolymer processing aid (Wendling 2003). The remainder was used in medical film coating and electronic applications involving printed circuit boards and precision bearings. 3M analytical studies of eight POSF-based product samples have identified the presence of PFOA at very low concentrations as a manufacturing impurity (Wendling 2003). In a biodegradation study of N-EtFOSE, PFOA could be formed instead of PFOS only under an abiotic or hydrolytic condition during the last step of the degradation pathway (Lange 2001). PFOA could also be an oxidation product or metabolite of the widely used telomer-based fluorochemicals manufactured by other companies (Hagen et al. 1981). PFHS, the sulfonate form of perfluorohexanesulfonyl fluoride (PHSF), could also be a residual by-product of POSF-based production. 3M also produced PHSF as a building block for compounds incorporated in fire-fighting foams and specific postmarket carpet treatment applications.

We also calculated the total organic fluorine value (TOF). TOF is the percentage of the molecular weight for each of the seven fluorochemicals that was attributed to organic fluorine (PFOS, 64.7%; PFHS, 61.9%; PFOA, 69.0%; PFOSAA, 55.3%; PFOSA, 64.7%; M570, 56.6%; M556, 58.1%) multiplied by the concentration measured for each fluorochemical and then summed across all seven fluorochemicals.

Sample Collection

Through cooperation with six American Red Cross blood banks, 645 serum samples (332 males, 313 females) were obtained from adult donors (20–69 years of age) in 2000–2001. Each sample (1–2 mL serum) was stored at –20°C in a plastic tube until laboratory analysis. Methodologic studies have not shown these fluorochemicals to be extracted from blood collection materials (Hansen K. Personal communication). The six American Red Cross blood banks represented donors from the following areas: Los Angeles, California; Portland, Oregon; Minneapolis–St. Paul, Minnesota; Charlotte, North Carolina; Hagerstown, Maryland; and Boston, Massachusetts. These metropolitan areas were chosen to represent different geographical locations in the United States. (The primary 3M POSF-based production facility in the United States was located in Decatur, AL, which is not in the area of any of these six locations. 3M did manufacture PFOA at its Cottage Grove, MN, plant, which is located in the southeastern part of the Minneapolis–St. Paul metropolitan area. At this site there was also some production of POSF-based materials but not to the same extent as manufactured at the Decatur plant.)

Approval for this study was obtained from the American Red Cross Biomedical Services Institutional Review Board. Samples were void of personal identifiers. The only known demographic factors were age, gender, and location. Each blood bank center was requested to provide approximately 100 serum samples: 10 samples for each 10-year age interval (20–29, 30–39, 40–49, 50–59, and 60–69) for each sex. We estimated *a priori* a statistical power of more than 95% to detect a 20% difference between any two 10-year age groups (combined locations) and more than 80% power to detect a 20% difference by sex within each age group.

Laboratory Assay

Tandem Labs (formerly Northwest Bioanalytical; Salt Lake City, UT) analyzed the fluorochemicals using techniques similar to those described by Hansen et al. (2001). Some modifications were made to the method to accommodate the small sample volume and to further assure the quality of the method for the analysis of human sera and plasma samples (Tandem Labs 1999, 2001a, 2001b).

Sample preparation. One hundred microliters of serum (or plasma) were extracted for analysis. The serum sample was added to 400 µL of 50 mM ammonium acetate in water in a polypropylene tube. Samples were vortex mixed and 50 µL of the internal standard, tetrahydroperfluorooctanesulfonate (THPFOS), was added to each sample before a second vortex mixing of approximately 30 sec. Three milliliters of methyl-*tert*-butyl ether (MTBE) was then added to each sample, which was vortex mixed for 30 sec and rotated for 10 min. The samples were centrifuged, and the MTBE layer was transferred to a clean polypropylene tube. The MTBE was evaporated to dryness, and the extract was reconstituted in 100 µL 30:70 (vol:vol) 20 mM ammonium acetate in water:20 mM ammonium acetate in methanol and vortex mixed for 15 sec. The samples were transferred to autovials and centrifuged at 3,000 rpm for 2 min.

Because of the difficulty in finding suitable human sera in the United States to use for method blanks and quality control (QC) samples, we obtained rural Chinese human plasma, collected in 1999, with low endogenous fluorochemical concentrations, from a source within the United States. Studies designed to characterize the selectivity and the extraction efficiency for the two matrices, conducted before the sample analysis, indicated that this plasma was a suitable choice for the calibration, blank, and QC matrix samples.

Blank samples were prepared in the same way as the sera samples. The internal standard solution was added to half of the blank plasma samples; the remaining plasma blanks were prepared without internal standard. The THPFOS internal standard was added to all plasma and serum samples at approximately 200 ppb. THPFOS is a partially fluorinated surfactant with a structure similar to the target analytes and is a reasonable surrogate for all the analytes in this study. Studies carried out before validation indicated that, because THPFOS has a retention time very similar to PFOA, extremely high levels of PFOA (> 500 ppb) can suppress the signal for THPFOS, resulting in artificially high reported levels of all analytes except PFOA. However, none of the samples analyzed in this study contained PFOA at high enough levels to suppress the THPFOS signal.

Calibration standards and QC samples, extracted from plasma, were prepared following the same procedure described for the samples except that the QC standards were spiked with a mixture of the seven target analytes concurrent with the addition of the internal standard.

Given the low-level presence of many of the target analytes in these biologic matrices, rigorous attention to the preparation, analysis, and data interpretation of blanks, calibration standards, and QC samples was critical. The analytical system was monitored for analytical artifacts such as carryover and for potential sources of contamination.

Analysis of samples. We used a Genesis Lightning C18 4-µm inner diameter, 2 × 50 mm analytical column (Argonaut Technologies, Inc., Foster City, CA) for chromatographic separation. The mobile phase was 20 mM ammonium acetate in water (a) and 20 mM ammonium acetate in methanol (b) and was programmed to run from 50% to 97.5% (b) over the course of a 9-min gradient. After a 2-min hold at 97.5% (b), the run equilibrated back to 50% (b) for 2 min. The flow rate was 300 µL/min and the column was maintained at 40°C. 2-(2-Methylethoxy)-ethanol (50 µL/min) was added postcolumn.

Extracts were analyzed on a Perkin-Elmer Sciex API3000 with a TurboIon Spray source (PE Sciex, Concord, Ontario, Canada) maintained at 400°C in the negative ion mode. All seven target analytes, along with the internal standard, were monitored in a single run recording a single transition for each ion with a dwell time of 70 msec. The collision energy was optimized for each analyte and ranged

from 25 to 80 eV. Because the focus of this work was quantitation of the total amounts of the target analytes, isomers of the target analytes were treated as a single peak even though full resolution was achieved. Quantitation was based on evaluation of the samples versus duplicate calibration curves extracted from plasma predetermined to contain little or no endogenous levels of the target analytes. The calibration curves were evaluated using a quadratic regression analysis (weighted $1/X^2$).

Method characterization and QC. Before analysis of the samples, we validated this method with respect to selectivity, accuracy, precision, quantitation range, and lower limit of quantitation (LLOQ). The extraction efficiency of the target analytes from human serum was between 15% and 70%, depending on the analyte. It was not necessary to correct for extraction efficiency because the calibration curve was extracted. The precision and accuracy of the method were determined for all analytes by analyzing three levels of QC samples in replicates of five in a single run. Intraassay precision was within 6% for all analytes; the accuracy was ± 18% for all analytes.

The LLOQ, evaluated for each run and analyte, varied between 1 and 4 ppb. We did not perform an experimental determination of the method LLOQ; instead, the lowest standard injected for a particular run that met the acceptance criteria was chosen as the LLOQ. Samples were evaluated quantitatively using a 6–8-point extracted calibration curve covering the target range for each analyte from about 1 to 500 ppb. For all analytes except PFOSA and PFOSAA, at least 75% of the back-calculated concentrations for the calibration standard was required to be within 15% (20% at LLOQ) of the theoretical concentration. For PFOSA and PFOSAA, at least 75% of the back-calculated concentrations for the calibration standard was required to be within 20% (25% at LLOQ) of the theoretical concentration.

We found that QC samples, calibration standards, and samples were stable through seven freeze–thaw cycles. All analytes were stable in matrix through 42 days at –20°C. All analytes were stable in the extracts through 7 days stored at room temperature.

Analytical QCs, extracted from plasma and prepared at the same time as the calibration curve, were injected intermittently during the analytical run. Three levels of QC samples, spanning the range of the method (~4, 150, and 400 ppb), were analyzed in duplicate. Analytical QCs were analyzed in duplicate; dilution QCs were also prepared and analyzed and run in triplicate for any assay that contained a diluted sample. QC samples were prepared at three levels spanning the calibration range: approximately 4, 150, and 400 ppb. The measured concentration for two-thirds of all analytical QCs was required to be within 20% (25% for PFOSA and PFOSAA) of the theoretical concentration, and no two QCs at the same concentration could be outside the limit. Any analytical run containing a diluted sample included a dilution QC, analyzed in triplicate for each dilution level. At least two of the dilution QCs were required to be within 20% of the theoretical concentration. Evaluation of QC samples injected during the analytical runs of the 645 samples indicated that the reported quantitative results may have varied ± 10% for precision and accuracy (Tandem Labs 2002).

Twenty-four samples were split and analyzed to provide an estimate of the reliability of the analyses conducted. The analytical laboratory was blind to the identity of these split samples. These analyses were performed concurrently with all other analyses of the study to minimize experimental error. Five split samples were analyzed from Charlotte, Los Angeles, Hagerstown, and Portland and four split samples from Boston. Inadvertently, no reliability analyses were performed on the Minnesota samples. There was a strong correlation between the split samples ($r = 0.9$) with PFOS, PFOA, and PFHS. The analysis of the split samples for the other fluorochemicals was problematic because only six split samples for PFOSAA and seven split samples for M570 had values that were above the LLOQ. None of the PFOSA and M556 split samples were above the LLOQ.

Data Analysis

We used measures of central tendency applicable to log-normally distributed data (median, geometric mean) for descriptive analyses. In those instances where a sample was measured below the LLOQ, the midpoint between zero and the LLOQ was used for calculation of the geometric mean. A sensitivity analysis of the assessment of this midpoint assumption and how it affected the calculation of the geometric mean was performed using the 10th and then 90th percentile values between zero and the LLOQ for those samples < LLOQ. Results for the geometric mean calculations for each fluorochemical remained similar to when the midpoint assumption was used (data not shown).

The log-linear relation between PFOS and PFOA was modeled as follows:

$$\ln[\text{PFOS}] = (a \times \ln[\text{PFOA}]) + (c \times \text{age}) + (d \times \text{sex}) + (f \times \text{age} \times \text{sex}) + g + \varepsilon, \quad [1]$$

where g is the intercept and ε is the error term. We used an analogous log-linear model to relate PFOS to PFHS. The interaction term between age and sex was not a significant predictor in either model and was therefore not included in the final analyses. Residuals were inspected to assure model assumptions provided reasonable fit.

To examine the relationship between PFOS concentration and the concentration of the two other precursor molecules, PFOSAA and M570, a nonlinear model was fit to the data:

$$\ln[\text{PFOS}] = \ln([\text{PFOSAA}]^a + [\text{M570}]^b) + (c \times \text{age}) + (d \times \text{sex}) + (f \times \text{age} \times \text{sex}) + g + \varepsilon. \quad [2]$$

This model represents an additive relation between PFOS concentration and the concentrations of the other two molecules because the hypothesized mechanisms of association—that is either correlated exposure sources or conversion from one molecule to another—suggested additivity rather than a multiplicative relation. At the same time, the model is consistent with the simpler models relating PFOS concentration to that of a single molecule and preserves the log-linear relationship of PFOS concentration to age and sex suggested by inspection of the residuals in these simpler models. The adjusted log-linear models were fit using maximum likelihood using the *lm* program, and the adjusted nonlinear model was fit using weighted nonlinear least squares, as implemented in the *nls* program; both are programs in S-Plus, version 6.0 (Insightful Corporation 2001). To avoid making normality assumptions in these log-linear models, bootstrapping was used to form confidence intervals for the parameters (Efron and Tibshirani 1993). In this method, a large number of full-size samples of the original observations are drawn with replacement, from each of which an estimate of the percentile is generated. The distribution of these estimates mimics the underlying sampling distribution for the original estimate assuming that the parent population looks like the sample. We used bias-corrected, accelerated percentiles to minimize residual bias. A bias correction factor is derived by comparing empirical percentiles with bootstrap percentiles, and acceleration is accomplished by partial jackknifing, a method of systematically resampling the data. The effect on parameter estimates and confidence intervals of including location as a random effect in the above models (using the S-Plus programs *lme* and *nlme*) was negligible; only the results from the nonhierarchical models are given. In order to minimize parametric assumptions in the estimation of extreme percentiles of the population, the bias-corrected, accelerated bootstrap method was also used to generate confidence intervals around the empirical percentiles for serum concentrations.

Results

The frequency distributions of PFOS, PFOA, PFHS, PFOSAA, and M570 are shown in Figure 1. Although the graphs suggest

log-normal distributions, only the PFOS distribution met such criteria based on the Shapiro-Wilk test. This lack of log normality is due to the greater percentage of subjects with values < LLOQ for PFOA, PFHS, PFOSAA, and M570. Statistical analyses are not presented for PFOSA and M556 because of the low number of subjects (n = 13) whose serum concentrations of PFOSA or M556 exceeded the LLOQ. The LLOQs for PFOSA and M556 ranged between 1.0 and 3.2 ppb. Although PFOSA and M556 are not presented in the later analyses, they were included in the calculation of the TOF value assuming the midpoint between zero and the LLOQ.

The range, interquartile range (IQR; i.e., the lower end of the second quartile and the upper end of the third quartile), number of samples < LLOQ, 90th percentile, median, geometric mean, and 95% confidence interval (CI) of the geometric mean for PFOS, PFOA, PFHS, PFOSAA, and M570 are provided in Table 1. The midpoint assumption was based on the LLOQs in place for the specific sample runs. No donor sample had more than one LLOQ. The percentages of samples < LLOQ for each fluorochemical were PFOS, 0.2%; PFOA, 8%; PFHS, 48%; PFOSAA, 58%; and M570, 60%.

For all donors, the geometric mean PFOS level was 34.9 ppb (95% CI, 33.3–36.5). The range of PFOS values was < LLOQ (4.3 ppb) to 1656.0 ppb. Serum samples from male subjects had significantly (p < 0.05) higher geometric means for PFOS than for serum samples from female subjects (Table 1). Serum

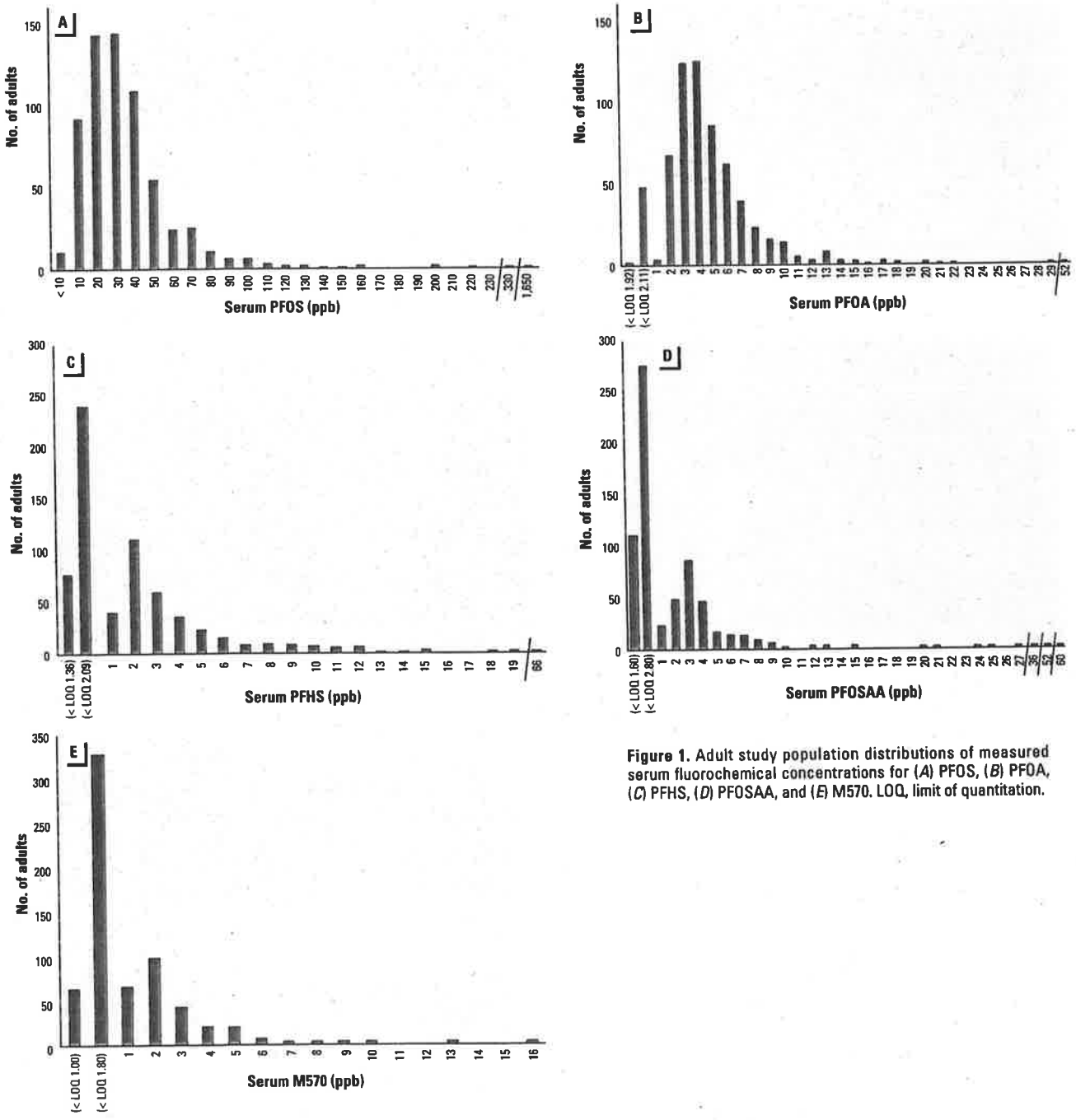

Figure 1. Adult study population distributions of measured serum fluorochemical concentrations for (*A*) PFOS, (*B*) PFOA, (*C*) PFHS, (*D*) PFOSAA, and (*E*) M570. LOQ, limit of quantitation.

samples from males also had significantly higher serum levels of PFOA and PFHS compared with serum samples from females, although the mean levels for both sexes were approximately one order of magnitude lower than that of PFOS. The overall geometric mean for the calculated TOF was 31.7 ppb (95% CI, 30.4–33.0; data not shown). The calculated TOF values ranged from 5.7 ppb to 1083.2 ppb.

Age was not an important predictor of adult serum fluorochemical concentrations (Figure 2). Instances of many outliers (e.g., M570 concentrations in males 40–49 and 60–69 years of age) occurred as a result of a large percentage of values < LLOQ that were within the 1.5 × interquartile range.

The unadjusted (for age and sex) serum PFOS concentrations were higher in Charlotte compared with the other locations as a consequence of a higher interquartile range (Table 2). Table 3 shows the results from the statistical bootstrap analysis that calculated mean serum fluorochemical values for each of the six locations adjusted for 10-year age intervals, sex, and their interaction terms. There was a 10-ppb difference in the adjusted mean serum PFOS concentration between the highest (Charlotte) and lowest (Boston) locations. The mean values for the other fluorochemicals were comparable between locations. Because PFOS is the primary contributor to the calculated TOF, the bootstrap analysis findings for TOF mirrored those of PFOS.

Scatter plots (log scale) between the five fluorochemicals are displayed in Figure 3. PFOS and PFOA were highly correlated (r = 0.63). PFOS had a lower correlation with PFOSAA (r = 0.42) and lower yet with M570 (r = 0.20). The correlation between PFOSAA and M570 was weak (r = 0.12). The remaining scatter plots display the correlations between PFOS and PFHS (r = 0.38) and PFOA and PFHS (r = 0.32).

Both PFOSAA and M570, adjusted for age and sex, were significant predictors of PFOS (Table 4). PFOSAA was the more significant of the two variables (PFOSAA t-value = 14.5; M570 t-value = 4.6). Controlling for age and sex, PFOA and PFHS were also associated with PFOS.

Table 5 presents the results from bootstrap analyses conducted to provide upper tolerance limits. The upper tolerance limits represent the concentration of each fluorochemical below which the stated proportion of the population is expected to be found. The biased corrected estimates for the 90th, 95th, and 99th percentile tolerance limits of the five serum fluorochemicals and TOF along with the upper limit (bound) from the 95% CI are shown in Table 5.

Discussion

The findings from this analysis of serum PFOS concentrations in 645 adult blood donors are consistent with previous, albeit sparse, human data (3M Company 2003; Hansen et al. 2001; Olsen et al. 2003b). Serum samples obtained in the United States during the late 1990s showed mean PFOS concentrations of 30 ppb in 18 pooled blood banks, 44 ppb from a pooled commercial sample of 500 donors, 33 ppb from a different pooled commercial sample of 200 donors, and 28 ppb in 65 commercial individual human sera samples (3M Company 2003; Hansen et al. 2001). These findings were also comparable to a limited number of serum samples from Europe that were determined to have mean serum PFOS concentrations at 17 ppb in five pooled samples from a blood bank in Belgium, 53 ppb in six pooled samples from the Netherlands, and 37 ppb from six pooled blood samples from Germany (3M Company, 2003). Mean liver and serum PFOS concentrations of 20.8 ng/g (ppb) and 18.2 ng/mL (ppb), respectively, were reported among 23 human donors with paired samples (Olsen et al. 2003b). The mean calculated TOF of 31.7 ppb in the present study is also consistent with the low–parts-per-billion TOF measurements of general population samples that have been reported since the 1960s (Belisle 1981; Singer and Ophaug 1979; Taves 1968; Taves et al. 1976).

Compared with these previous assessments, in the present study we analyzed a sufficient sample size that facilitated the characterization of the serum fluorochemical distribution by age and sex. This included the calculation of estimates of upper tolerance limits and their upper bounds. The highest serum PFOS measurement (confirmed by reassay of the sample) was 1,656 ppb. This PFOS sample approximated the average serum PFOS levels observed for POSF-related production workers (Olsen et al. 1999, 2003a); however, there is no POSF-related production plant in the Portland area. Because donor samples were anonymous, it is not possible to

Table 1. Measures of central tendency of serum fluorochemical concentrations (ppb) for American Red Cross blood donors (n = 645) by sex.

	PFOS	PFOA	PFHS	PFOSAA	M570
All (n = 645)					
Range	< LLOQ (4.3)–1656.0	< LLOQ (1.9)–52.3	< LLOQ (1.4)–66.3	< LLOQ (1.6)–60.1	< LLOQ (1.0)–16.4
IQR	24.7–48.5	3.4–6.6	< LLOQ (2.1)–3.4	< LLOQ (2.8)–3.4	< LLOQ (1.8)–2.2
< LLOQ (number)	< 4.3 (1)	< 1.9 (2)	< 1.4 (72)	< 1.6 (101)	< 1.0 (63)
		< 2.1 (48)	< 2.1 (235)	< 2.8 (271)	< 1.8 (326)
Cumulative 90%	70.7	9.4	6.3	5.2	3.8
Median	35.8	4.7	1.5	< LLOQ (2.8)	< LLOQ (1.8)
Geometric mean	34.9	4.6	1.9	2.0	1.3
95% CI geometric mean	33.3–36.5	4.3–4.8	1.8–2.0	1.9–2.1	1.3–1.4
Males (n = 332)					
Range	< LLOQ (4.3)–1656.0	< LLOQ (1.9)–29.0	< LLOQ (1.4)–66.3	< LLOQ (1.6)–60.1	< LLOQ (1.0)–16.4
IQR	28.3–49.7	3.6–7.0	< LLOQ (2.1)–3.8	< LLOQ (2.8)–3.3	< LLOQ (1.8)–2.2
< LLOQ (number)		< 2.1 (19)	< 1.4 (30)	< 1.6 (58)	< 1.0 (36)
			< 2.1 (104)	< 2.8 (146)	< 1.8 (163)
Cumulative 90%	72.6	10.1	7.9	4.7	3.5
Median	37.4	4.9	2.1	< LLOQ (2.8)	< LLOQ (1.8)
Geometric mean	37.8	4.9	2.2	1.9	1.3
95% CI geometric mean	35.5–40.3	4.6–5.3	2.0–2.4	1.8–2.1	1.2–1.4
Females (n = 313)					
Range	6.0–226.0	< LLOQ (2.1)–52.3	< LLOQ (1.4)–15.3	< LLOQ (1.6)–27.6	< LLOQ (1.0)–10.6
IQR	22.0–45.8	3.1–6.2	< LLOQ (2.1)–2.8	< LLOQ (2.8)–3.6	< LLOQ (1.8)–2.2
< LLOQ (number)		< 1.9 (2)	< 1.4 (42)	< 1.6 (43)	< 1.0 (27)
		< 2.1 (29)	< 2.1 (131)	< 2.8 (125)	< 1.8 (163)
Cumulative 90%	69.7	8.4	5.0	6.1	4.0
Median	31.3	4.4	< LLOQ (2.1)	< LLOQ (2.8)	< LLOQ 1.8)
Geometric mean	32.1	4.2	1.6	2.1	1.3
95% CI geometric mean	30.0–34.3	3.9–4.5	1.5–1.8	2.0–2.3	1.2–1.4

determine anything else about this individual besides sex (male), age (67 years), and location of the blood bank (Portland). The next highest donor level for PFOS was considerably lower at 329 ppb (also a male, 62 years of age, from the Portland blood bank location), and the next eight highest serum PFOS values (range, 139–226 ppb) were measured in four females and four males from the Charlotte (n = 4), Hagerstown (n = 2), Los Angeles (n = 1), and Minneapolis–St. Paul (n = 1) blood bank centers.

There was a relatively narrow range of geometric mean serum concentrations for the six locations studied. Because no exposure data were available, any explanation of the variation of serum concentrations once adjusted for age and sex, albeit small, between these locations would only be speculation. As discussed above, the pathways leading to the presence of PFOS in human blood are not well characterized but likely involve environmental exposure (3M Company 2003; Hansen et al. 2002; Martin et al. 2002; OECD 2002) to PFOS or to precursor molecules and residual levels of PFOS or PFOS precursors in industrial and commercial products (3M Company 2003).

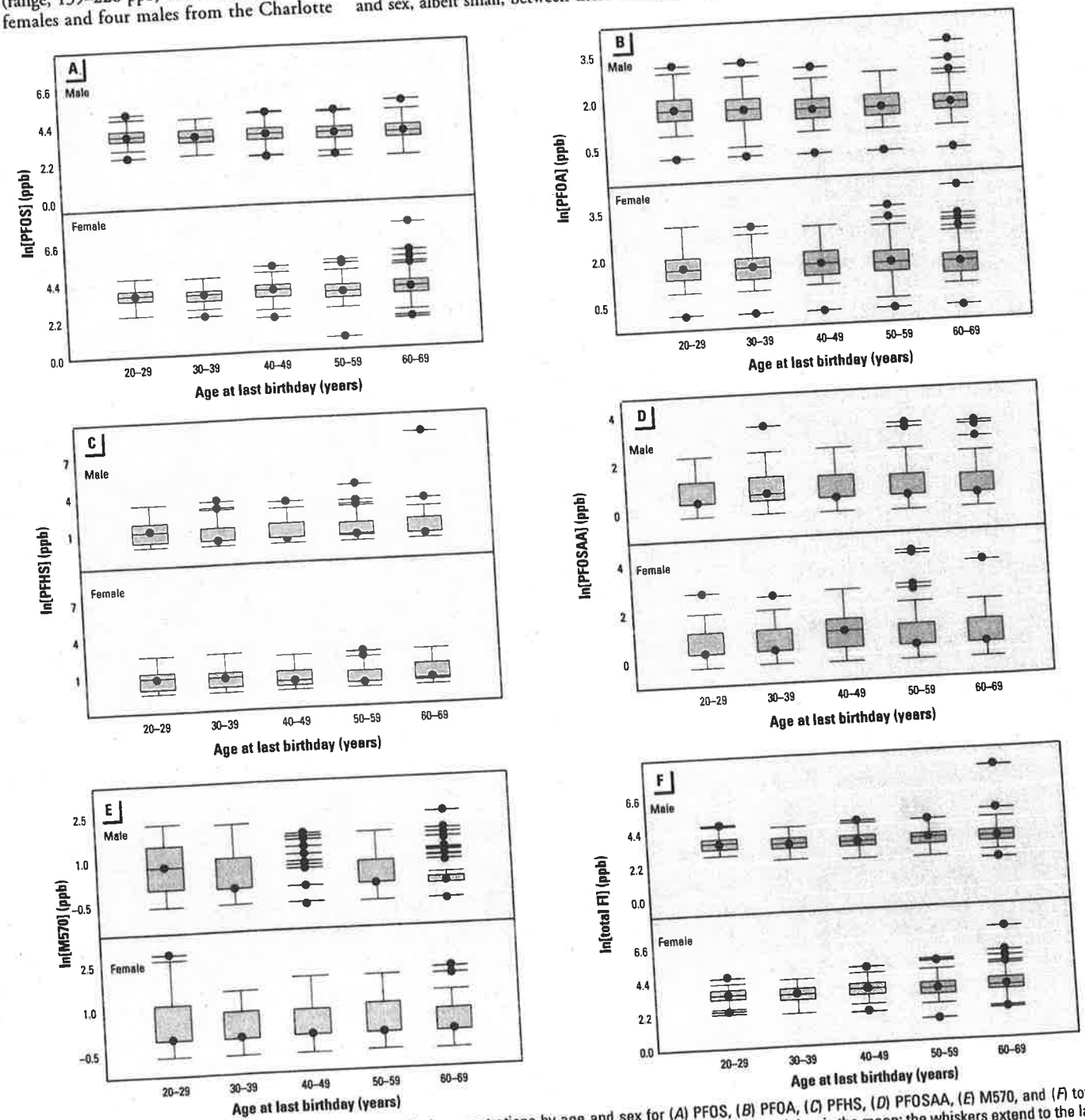

Figure 2. Box and whisker plots of serum fluorochemical concentrations by age and sex for (*A*) PFOS, (*B*) PFOA, (*C*) PFHS, (*D*) PFOSAA, (*E*) M570, and (*F*) total fluorochemicals. The boxes indicate the interquartile ranges of the natural log distributions; the circle within each box is the mean; the whiskers extend to the last observation within 1.5 times the interquartile range; and the circles outside the whiskers represent observations outside the 1.5 × interquartile range.

Our findings showed a correlation between PFOS and PFOA. PFOS has been measured in both human populations and wildlife, including marine mammals and piscivorous birds (3M Company 2003; Giesy and Kannan 2001; Hansen et al. 2001; Kannan et al. 2001a, 2001b, 2002a, 2002b). Serum PFOA concentrations, however, have been consistently quantified (i.e., measured above the LLOQs) primarily in humans. This association between PFOS and PFOA is of significant interest because PFOA cannot convert directly from PFOS (or vice versa). Whether this statistical association is due to the presence of PFOA as a by-product in POSF-related production or to other nonrelated environmental or consumer products (e.g., higher chain telomers) remains to be determined. Another unanswered question is whether perfluorooctanesulfonamides can metabolize in humans to PFOA.

PFOS was also correlated with two fluorochemicals, PFOSAA and M570. These analytes may come from oxidation of compounds used in consumer products involving paper/packaging and carpet/textile/upholstery protectants, respectively.

As with any interpretation of data obtained from a study population, questions arise regarding how well the data represent the source population and the ability to generalize from the information collected. We believe the donor selection process used in this study likely resulted in a reasonable representation of the overall donor population that was providing blood at the time when these donors were sampled. Donors were not specifically informed of the fluorochemicals assayed, nor was there any linkage between the sample collected and personal identifiers. No information was obtained about past exposure histories to POSF-related chemistries and materials or the other fluorochemicals that were analyzed. Unlike other biomonitoring efforts of environmental chemicals [Centers for Disease Control and Prevention (CDC) 2003], it was not the intent of this study design to be representative of the diverse U.S. general adult population. American Red Cross blood donors are a self-selected group from the U.S. population (Allen and Butler 1993; Andaleeb and Basu 1995; Burnett 1982; Chiavetta et al. 2000; Leibrecht et al. 1976; Oswalt 1977; Wood et al. 2001). Motivations to donate blood may include altruism, desire for personal credit, low self-esteem, and social pressure. Donors in this study did not include children or the elderly. Future biomonitoring efforts by the National Center for Environmental Health at the CDC may analyze for PFOS and other fluorochemicals that will allow for a representative sample analysis of the U.S. civilian population (Calafat A. Personal communication).

A large body of toxicology and epidemiology data is available for review regarding PFOS (3M Company 2003; OECD 2002). Briefly, PFOS concentrates primarily in the liver and, to a lesser extent, in the plasma of rats (Johnson et al. 1979). There appears to be significant enterohepatic circulation of PFOS with both urinary and fecal excretion (Johnson et al. 1984). Studies in rodents and primates suggest toxicity varies with cumulative dose of PFOS (Butenhoff et al. 2002b; Butenhoff and Seacat 2001; Grasty et al. 2002; Lau et al. 2003; Seacat et al. 2002b, 2002c; Thibodeaux et al. 2003; Thomford et al. 2002). Lowered serum total cholesterol appeared to be a consistent early finding, with cumulative toxicity resulting in metabolic wasting and ultimately death in laboratory animals exposed to high doses. In cynomolgus monkeys, serum cholesterol levels began to decline above 100 ppm serum PFOS concentrations (Seacat et al. 2002c). The serum half-life of elimination approximated 200 days at the end of dosing (180 days, 0.75 mg/kg/day). An interim analysis estimated the half-life of serum elimination for PFOS in humans to be approximately 9 years (standard deviation = 6) based on nine POSF-related production workers (Burris et al. 2002). A 2-year feeding study of PFOS in rats produced a modest liver tumor response in the high-dose group (20 ppm PFOS in feed) (Seacat et al. 2002a). A 2-year feeding study of N-EtFOSE provided comparable findings (Thomford et al. 2002). PFOS and N-EtFOSE are not genotoxic in standard tests (3M Company 2003). The mechanisms of toxicity are not fully understood but may include effects on fatty acid transport and metabolism, membrane function, and/or mitochondrial bioenergetics (Berthiaume and Wallace 2002; Hu et al. 2002; Luebker et al. 2002; O'Brien and Wallace 2002; Starkov and Wallace 2002). PFOS caused modest peroxisome proliferation in rats (Berthiaume and Wallace 2002; Seacat et al. 2002b). Medical surveillance of POSF production employees has not been associated with adverse clinical chemistry or hematology results (Olsen et al. 1999, 2003a). A retrospective cohort mortality study of this production workforce reported an excess of bladder cancer based on three deaths compared with 0.2 expected (Alexander et al. 2003). It was not determined whether this association was PFOS-related or possibly due to other occupational exposures or nonoccupational exposures. Whether this is a biologically plausible association with PFOS is questionable, as PFOS did not produce bladder or other urinary tract toxicity in rat or primate chronic or subchronic studies (Seacat et al. 2002a, 2002b, 2002c). PFOS is not likely to be insoluble in human urine (solubility, 305 µg/mL at 23–24°C) at the serum concentrations present in these workers (Ellefson 2001). In a review article, Cohen (1998) reported that all of the chemical agents listed as known bladder cancer agents were genotoxic and had metabolites that were genotoxic or precipitated in the urine.

Table 2. Measures of central tendency of serum PFOS concentrations (ppb) by the six American Red Cross blood bank locations.

	Los Angeles (n = 125)	Portland (n = 107)	Minneapolis–St. Paul (n = 100)	Charlotte (n = 96)	Hagerstown (n = 108)	Boston (n = 109)
Range	6.6–205.0	6.0–1656.0	7.7–207.0	19.3–166.0	7.6–226.0	< LLOQ (4.3)–87.2
IQR	29.5–53.7	17.2–37.7	23.9–43.3	36.3–70.9	24.4–48.1	20.8–39.1
90th percentile	70.1	49.4	71.7	105.3	69.8	48.7
Median	42.2	26.0	31.7	48.9	35.7	29.5
Geometric mean	40.4	27.0	33.1	51.5	35.3	28.0
95% CI geometric mean	37.0–44.0	23.5–31.1	29.8–36.7	46.8–56.8	31.8–39.2	25.4–31.0

Table 3. Age- and sex-adjusted mean and 95% CIs of serum fluorochemical concentration (ppb) for the six American Red Cross blood bank locations.

	PFOS Mean (95% CI)	PFOA Mean (95% CI)	PFHS Mean (95% CI)	PFOSAA Mean (95% CI)	M570 Mean (95% CI)	TOF Mean (95% CI)
Los Angeles	35.0 (33.4–36.5)	4.6 (4.4–4.8)	1.9 (1.8–2.0)	2.0 (1.9–2.2)	1.3 (1.3–1.4)	30.2 (29.0–31.3)
Portland	32.8 (30.5–34.2)	4.3 (4.0–4.4)	1.8 (1.7–2.0)	2.1 (2.0–2.3)	1.3 (1.2–1.4)	28.5 (26.7–30.4)
Minneapolis–St. Paul	34.8 (31.9–36.3)	4.5 (4.1–4.7)	1.8 (1.6–2.0)	1.8 (1.7–2.1)	1.3 (1.1–1.4)	29.7 (27.4–32.1)
Charlotte	39.0 (36.2–40.7)	5.0 (4.6–5.1)	2.2 (2.0–2.4)	2.1 (1.9–2.4)	1.4 (1.3–1.5)	33.4 (31.3–35.7)
Hagerstown	34.9 (32.8–36.5)	4.5 (4.2–4.6)	1.9 (1.8–2.1)	1.9 (1.8–2.1)	1.3 (1.2–1.4)	30.1 (28.4–31.9)
Boston	29.0 (26.0–30.3)	5.3 (4.6–5.6)	1.9 (1.7–2.3)	1.6 (1.4–1.8)	1.3 (1.2–1.5)	26.4 (23.9–29.2)

Given the consistency of the data analyzed, we hypothesize that the average serum PFOS concentrations in nonoccupational adult populations may range from 30 to 40 ppb, with 95% of a population's serum PFOS concentrations below 100 ppb. After consideration of serum and liver PFOS concentrations associated with no-observed-adverse-effect levels (NOAELs) and those associated with benchmark dose values from various toxicology studies, protective values [i.e., benchmark internal concentrations (BMICs)] were chosen as "points of departure" for risk characterization (3M Company 2003). For serum PFOS comparisons, the lower 95% confidence limit of the BMIC (5% response), 31 ppm, was chosen based on rat pup weight gain during lactation (3M Company 2003). Although rat pup weight gain was considered the most sensitive end point, comparisons were also made for other end points. For liver toxicity, a serum PFOS concentration of 44 ppm was associated with the NOAEL (3M Company 2003; Seacat et al. 2002b). For liver tumors in male and female rats, the lower 95% confidence limit of the BMIC (10% response), 62 ppm, was calculated (3M Company 2003). Therefore, for these points of departure, a human serum concentration of 100 ppb (the upper bound estimate of the 95th percentile observed in the present study) was associated with margins of exposure of 310, 440, and 620, respectively (3M Company 2003). When interpreting the significance of these margins of exposure, it should be noted that they are based on the use of an internal dose (concentration) of a compound that is not metabolized and poorly eliminated. These facts reduce the uncertainty in considering intraspecies or interspecies response variability based on either toxicokinetic or toxicodynamic factors.

PFOA was the other fluorochemical assayed in the present study for which there are considerable toxicology and epidemiology data (Kennedy et al. In press). Sex and species differences exist in the elimination of PFOA. PFOA is rapidly cleared (hours) in female rats compared with clearance in approximately 1 week in male rats (DuPont Haskell Laboratory 1982; Hanhijarvi and Ylinen 1988; Johnson and Ober 1980). In addition

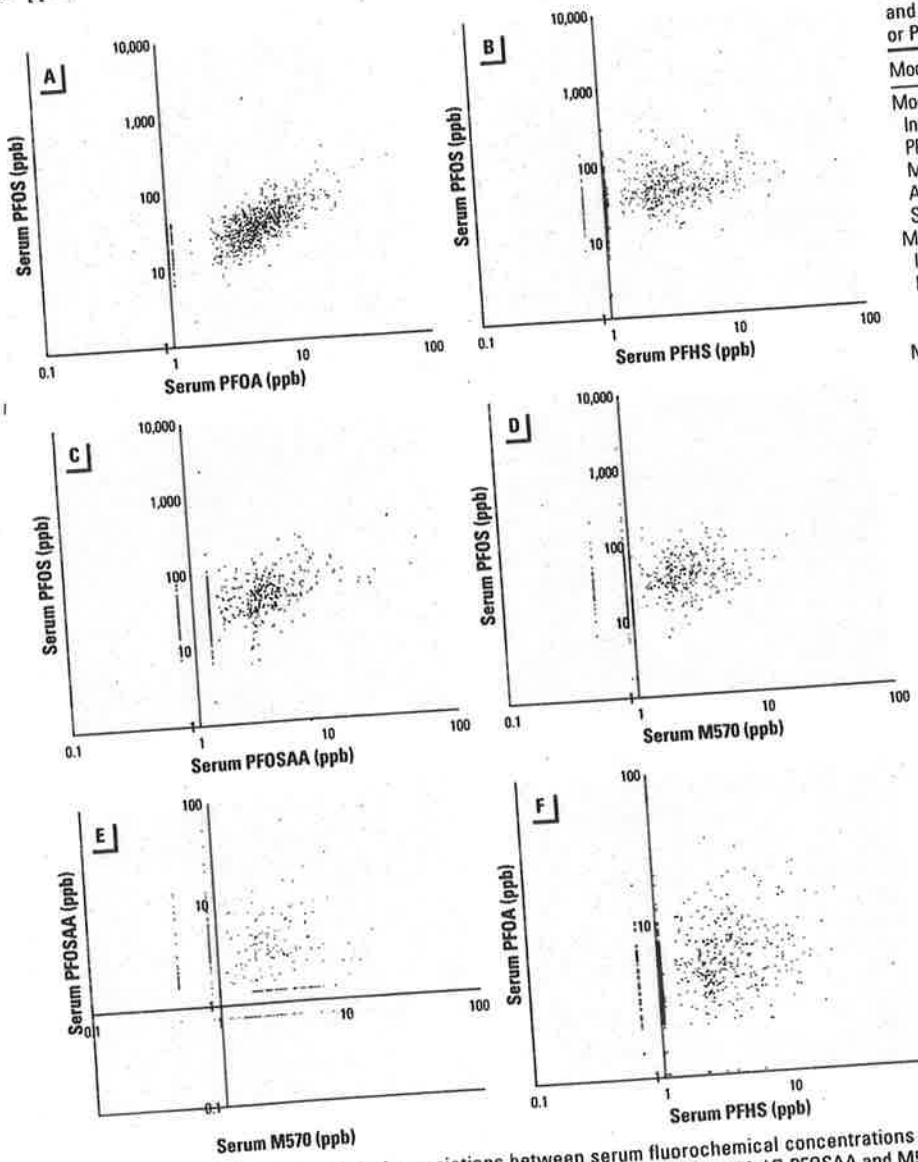

Figure 3. Scatter plots (log scale) of associations between serum fluorochemical concentrations for (*A*) PFOS and PFOA, (*B*) PFOS and PFHS, (*C*) PFOS and PFOSAA, (*D*) PFOS and M570, (*E*) PFOSAA and M570, and (*F*) PFOA and PFHS.

Table 4. Relationship between PFOS concentration and PFOSAA and M570 (Model 1), PFOA (Model 2), or PFHS (Model 3) adjusted for age and sex.

Model	Coefficient	95% CI
Model 1[a]		
Intercept	2.323	2.167–2.481
PFOSAA	0.544	0.473–0.619
M570	0.288	0.187–0.389
Age	−0.011	−0.039–0.020
Sex	0.198	0.116–0.282
Model 2[b]		
Intercept	2.575	2.412–2.723
PFOA	0.591	0.527–0.655
Age	−0.010	−0.010–0.016
Sex	0.074	0.005–0.143
Model 3[c]		
Intercept	3.252	3.073–3.408
PFHS	0.267	0.213–0.317
Age	−0.008	−0.030–0.029
Sex	0.087	0.017–0.181

[a] $\ln[PFOS] = \ln([PFOSAA]^a + [M570]^b) + (c \times age) + (d \times sex) + g + \varepsilon$, where the serum concentration of the fluorochemical is shown in brackets and ε is the error term. [b] $\ln[PFOS] = \ln[PFOA]^a + (c \times age) + (d \times sex) + g + \varepsilon$. [c] $\ln[PFOS] = \ln[PFHS]^a + (c \times age) + (d \times sex) + g + \varepsilon$.

Table 5. Estimates (ppb) of upper tolerance limits and their upper 95% confidence limits for five serum fluorochemicals and a calculated TOF value.

Fluorochemical	Upper tolerance limit	Estimate	Upper 95% confidence limit
PFOS	90%	70.7	74.3
	95%	88.5	100.0
	99%	157.3	207.0
PFOA	90%	9.4	10.1
	95%	12.1	13.6
	99%	19.8	25.8
PFHS	90%	6.3	7.0
	95%	9.5	10.8
	99%	17.0	22.4
PFOSAA	90%	5.3	5.9
	95%	7.6	8.5
	99%	19.4	27.6
M570	90%	3.7	4.0
	95%	5.0	5.4
	99%	8.1	10.3
TOF	90%	59.9	63.1
	95%	75.1	80.9
	99%	137.3	187.5

to urinary excretion, biliary excretion and reabsorption of PFOA occur (Johnson et al. 1984). In the primate, the terminal phase elimination half-life in serum for both sexes was approximately 1 month (Butenhoff et al. 2002a). An interim analysis estimated the half-life of serum elimination for PFOA in humans to be approximately 4 years (standard deviation, 4) (Burris et al. 2002). The liver is a primary target organ for toxicity. In rats, administration of the ammonium salt of PFOA (APFO) resulted in peroxisome proliferation, uncoupling of mitochondrial oxidative phosphorylation, increased mitochondrial DNA copy number, and altered lipid metabolism (Berthiaume and Wallace 2002; Haughom and Spydevold 1992; Keller et al. 1992). Rats fed a diet of 300 ppm PFOA (daily dose of 15 mg/kg/day) had increased incidences of liver, Leydig cell, and pancreas acinar cell adenomas (Biegel et al. 2001). The tumors likely occurred via nongenotoxic mechanisms. Peroxisome proliferation may account for the increase in liver tumors. Enhanced hepatic aromatase activity resulting in increased estradiol may account for the Leydig cell tumors (Biegel et al. 1995, 2001; Cook et al. 1992, 1999). The pancreas acinar cell tumors were hypothesized to be a result of a mild but sustained increase in plasma cholecystokinin levels secondary to hepatic cholestasis (Biegel et al. 2001; Obourn et al. 1997). Changes in plasma cholecystokinin have not been observed in primates or humans exposed to APFO (Butenhoff et al. 2002a; Olsen et al. 2000). Hepatic toxicity, hypolipidemia, and abnormal hormone levels have not been associated with serum PFOA concentrations in APFO production workers whose serum levels have averaged 5 ppm with a range of and 0.1–114 ppm (Gilliland and Mandel 1996; Olsen et al. 1998, 2000). A retrospective cohort mortality study of APFO production workers did not report statistically significant increased standardized mortality ratios for liver cancer, cirrhosis of the liver, or pancreas cancer, although an association was observed with prostate cancer mortality and nonspecific job duration of at least 10 years in the chemical plant (Gilliland and Mandel 1993). This association with prostate cancer mortality, however, was not observed in an updated study that used an exposure matrix specific to APFO production activities (Alexander 2001).

Correction

Table 4 was accidently omitted from the original manuscript published online and has been added here.

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Exposure to Perfluorooctane Sulfonate during Pregnancy in Rat and Mouse. I: Maternal and Prenatal Evaluations

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The maternal and developmental toxicities of perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$) were evaluated in the rat and mouse. PFOS is an environmentally persistent compound used as a surfactant and occurs as a degradation product of both perfluorooctane sulfonyl fluoride and substituted perfluorooctane sulfonamido components found in many commercial and consumer applications. Pregnant Sprague-Dawley rats were given 1, 2, 3, 5, or 10 mg/kg PFOS daily by gavage from gestational day (GD) 2 to GD 20; CD-1 mice were similarly treated with 1, 5, 10, 15, and 20 mg/kg PFOS from GD 1 to GD 17. Controls received 0.5% Tween-20 vehicle (1 ml/kg for rats and 10 ml/kg for mice). Maternal weight gain, food and water consumption, and serum chemistry were monitored. Rats were euthanized on GD 21 and mice on GD 18. PFOS levels in maternal serum and in maternal and fetal livers were determined. Maternal weight gains in both species were suppressed by PFOS in a dose-dependent manner, likely attributed to reduced food and water intake. Serum PFOS levels increased with dosage, and liver levels were approximately fourfold higher than serum. Serum thyroxine (T_4) and triiodothyronine (T_3) in the PFOS-treated rat dams were significantly reduced as early as one week after chemical exposure, although no feedback response of thyroid-stimulating hormone (TSH) was observed. A similar pattern of reduction in T_4 was also seen in the pregnant mice. Maternal serum triglycerides were significantly reduced, particularly in the high-dose groups, although cholesterol levels were not affected. In the mouse dams, PFOS produced a marked enlargement of the liver at 10 mg/kg and higher dosages. In the rat fetuses, PFOS was detected in the liver but at levels nearly half of those in the maternal counterparts, regardless of administered doses. In both rodent species, PFOS did not alter the numbers of implantations or live fetuses at term, although small deficits in fetal weight were noted in the rat. A host of birth defects, including cleft palate, anasarca, ventricular septal defect, and enlargement of the right atrium, were seen in both rats and mice, primarily in the 10 and 20 mg/kg dosage groups, respectively. Our results demonstrate both maternal and developmental toxicity of PFOS in the rat and mouse.

Key Words: perfluorooctane sulfonate; maternal; prenatal; toxicity; rodent.

Organic fluorochemicals are compounds in which one or more carbon-hydrogen (C-H) bond is replaced by a carbon-fluorine (C-F) bond. These C-F bonds are one of the strongest in nature and contribute to the unique stability of fluorochemicals in the environment, even at high temperatures. In perfluorinated compounds, all of the C-H bonds are replaced by C-F bonds (Kissa, 1994). When these compounds are mixed with hydrocarbons and water, three immiscible phases are formed, indicating that the perfluoroalkanes are both oleophobic and hydrophobic. By adding a charged moiety (such as a sulfonic acid) to a perfluorinated carbon chain, the chemical molecule becomes more water soluble, resulting from the hydrophilic nature of the added functional group. These amphoteric perfluorinated organic chemicals are used in commerce principally for their surfactant properties.

Perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$) is a perfluorinated alkane with a sulfonyl group. The intermediate precursor, perfluorooctane sulfonyl fluoride, provides a link to products with other functional groups, such as free acids, metal salts, sulfonyl halides, and sulfonamides. Since the 1950s, with the commercial scale-up of electrochemical fluorination, PFOS and other perfluorinated organic compounds that metabolize into PFOS (as an end-stage metabolite and breakdown product) have been used in a wide variety of industrial and consumer applications that include stain-resistant coatings for fabrics and carpets, oil-resistant coatings for paper products approved for food contact, fire-fighting foams, mining and oil well surfactants, floor polishes, and insecticide formulations (Renner,

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2001; Seacat *et al.*, 2002). In all, PFOS or products degrading to PFOS are used in over 200 products and applications. However, 3M Company, the primary manufacturer of these compounds and products, discontinued production at the end of 2002.

The widespread use of PFOS and its related products, as well as the environmental stability of the perfluorinated organic chemical, have led to documentation of its presence in both human and wildlife populations worldwide (Giesy and Kannan, 2001; Hansen *et al.*, 2001; Kannan *et al.*, 2001a,b; 2002a,b,c; Olsen *et al.*, 2001a,b,c). Olsen *et al.* (1999) reported an average serum PFOS level of 2 ppm in 3M production workers, with 5% of them having levels ≥ 6 ppm. Current information from a broad survey of individual blood samples from adult Red Cross blood donors, children (ages 2–12) from a streptococcal A clinical trial, and a group of elderly subjects enrolled in a longitudinal study of cognitive function indicates that the upper bound of the 95th percentile serum concentration is approximately 100 ppb, with a mean of approximately 30–40 ppb (Olsen *et al.*, 2001a,b,c). Recent studies by Giesy and coworkers (Giesy and Kannan, 2001; Kannan *et al.*, 2001a,b; 2002a,b,c) reported detection of PFOS in a variety of wildlife species, including fresh-water and marine mammals, fishes, birds, and shellfish. Although distribution of the chemical appears to be global, including remote locations in the Arctic and North Pacific Oceans, concentrations of PFOS in these animals are relatively greater in the more populated and industrial regions. These investigators also suggested that PFOS can be biomagnified in the top levels of the food chain.

Pharmacokinetic studies have shown that PFOS is readily absorbed, distributed, and accumulated in the serum and liver but poorly eliminated (urinary and fecal excretion half-life estimated at >90 days in the rat; Johnson and Ober, 1979; Johnson *et al.*, 1979, 1984; Seacat *et al.*, 2003). In the rat, a serum elimination half-life of 7.5 days was reported after an oral treatment of PFOS (Johnson *et al.*, 1979); in Cynomolgus monkeys, a half-life of 200 days was described (Seacat *et al.*, 2002); and in humans, a mean half-life of approximately 8.7 years was recently estimated from retired production workers (Geary Olsen, 3M, 2002, personal communication). The potential mammalian toxicity of PFOS has been investigated. In the rat, reduction of body weight, liver hypertrophy, and decreased serum cholesterol and triglycerides have been reported after subchronic exposure to PFOS (Seacat *et al.*, 2003). PFOS has been suggested to interfere with mitochondrial bioenergetics, gap junctional intercellular communication, and fatty acid-protein binding in the liver (Berthiaume and Wallace, 2002; Hu *et al.*, 2002; Luebker *et al.*, 2002a; Starkov and Wallace, 2002). In addition, PFOS-induced hepatic peroxisome proliferation has been indicated in both rat and mouse (Berthiaume and Wallace, 2002; Haughom and Spydevold, 1992; Sohlenius *et al.*, 1993). Seacat and coworkers (2002) have evaluated PFOS toxicity in Cynomolgus monkeys and reported weight loss, hepatocellular hypertrophy, and lipid vacuolation, as well as reductions in serum cholesterol, triiodothyronine, and estradiol. The potential reproductive and developmental toxicities of PFOS have not been fully elaborated. Case *et al.* (2001) examined the effects of PFOS in rabbits and noted reductions of maternal weight gain and fetal weight at 3.75 mg/kg and higher dosage but no significant incidence of malformation. More recently, 3M completed a multigenerational reproduction study with PFOS in rats (Butenhoff *et al.*, 2002), which did not indicate any adverse effects on mating and fertility; however, significant reductions of body weight and perinatal viability were noted.

In light of the prevalence and persistence of PFOS in both humans and wildlife, this study was undertaken to provide a comprehensive evaluation of the developmental toxicity of this fluorochemical. This article summarizes the observations from the pregnant dams and term fetuses, and a companion article (Lau *et al.*, 2003) will address the postnatal findings in rats and mice.

MATERIALS AND METHODS

Chemicals

Perfluorooctane sulfonate (PFOS, potassium salt; 91% pure) was purchased from Fluka Chemical (Steinheim, Switzerland). Our analysis indicated that approximately 71% of the chemical was straight-chain, and the remaining 29% was branched. Additional chromatographic analysis indicated that the chemical obtained from Fluka appeared to be the same material produced by the 3M Company (St. Paul, MN) and tested in the earlier developmental and reproductive studies by 3M.

Animal Treatment

Timed-pregnant Sprague-Dawley rats and CD-1 mice obtained from Charles River Laboratories (Raleigh, NC) were bred within a 4-h period and overnight, respectively. Those animals with spermatozoa in a vaginal smear and/or with a copulatory plug were considered to be at gestational day (GD) 0. In a separate study, mature female rats weighing 200 g were obtained from the same supplier. Animals were housed individually in polypropylene cages with heat-treated pine shavings for bedding and provided pellet chow (LabDiet 5001, PMI Nutrition International, Brentwood, MO) and tap water *ad libitum*. Animal facilities were controlled for temperature (20–24°C) and relative humidity (40–60%), and operated under a 12-h light-dark cycle.

Rats. PFOS was freshly prepared daily at 1, 2, 3, 5, and 10 mg/ml of 0.5% Tween-20 vehicle and administered to the pregnant dams by gavage at a volume of 1 ml/kg/day from GD 2 through GD 20. Controls received vehicle alone. Throughout gestation and treatment, maternal body weights as well as food and water consumption were recorded. Blood from each dam was collected between 9–11 A.M. on GDs 7 and 14 from the lateral tail vein and on GD 21 after decapitation. Aliquots of serum from these blood samples were stored at −20°C for subsequent analysis of PFOS concentration, thyroid hormones, corticosterone, prolactin, cholesterol, and lipid content. On GD 21, both maternal and some fetal livers were removed, weighed, and immediately frozen on dry ice and stored at −80°C for PFOS analysis. For other animals, the gravid uterus was removed and examined, and individual live fetuses were weighed and prepared for teratological evaluation.

In a separate study, adult female rats were given either 3 or 5 mg/kg PFOS daily for 20 days; controls received the Tween-20 vehicle. Blood samples were withdrawn from tail vein at 3, 7, and 14 days after the initiation of PFOS exposure, and trunk blood was obtained from decapitation after 20 days of

chemical treatment. Serum samples were prepared and stored at –20°C for T_3, T_4, and thyroid-stimulating hormone (TSH) analyses.

Mice. PFOS (0.1, 0.5, 1.0, 1.5, and 2.0 mg/ml vehicle) was similarly prepared and administered by gavage at a volume of 10 ml/kg/day from GD 1 through GD 17. Maternal weight as well as food and water consumption were monitored throughout gestation. Some mice were sacrificed on GDs 6 and 12 by CO_2 asphyxiation. The remaining dams were sacrificed on GD 18. Blood was collected from the descending aorta, and serum samples were prepared and analyzed for PFOS concentration as well as lipid content. On GD 18, maternal livers from representative animals were dissected, weighed, and immediately frozen on dry ice and stored at -80°C for PFOS analysis. For other animals, the gravid uterus was removed and examined, and individual live fetuses were weighed and prepared for teratological evaluation.

Teratological Evaluation

The gravid uterus of the pregnant rat or mouse was removed and weighed; the numbers and positions of the live or dead fetuses, as well as resorptions, were recorded. Live fetuses were weighed individually, gender-determined, and examined for external abnormalities. Half of the fetuses were prepared for skeletal examination, and the other half were prepared for visceral evaluation.

Skeletal evaluation. Fetuses were killed with an overdose of pentobarbital, eviscerated, and fixed in 95% ethanol. Specimens were subsequently stained with Alizarin red and Alcian blue to visualize bone and cartilage, respectively. Skeletal morphology was evaluated as described previously (Narotsky and Rogers, 2000).

Visceral evaluation. Fetuses were fixed in Bodian's solution (2% formaldehyde, 5% acetic acid, 72% ethanol, 21% water). Examination of the head, thoracic, and abdominal viscera were carried out using a freehand razor dissection.

Serum Chemistry

Serum samples were analyzed for total cholesterol, triglycerides, sorbitol dehydrogenase, glucose, bile acids, and total bilirubin levels using a Cobas Fara II chemistry analyzer (Roche Diagnostics, Basel, Switzerland).

Radioimmunoassays

Concentrations of serum hormones were derived from a standard curve encompassing a range of reference standards specific for each assay. If the value of unknown fell above or below this range, it was arbitrarily assigned the values of the highest or lowest reference standard. Internal standards from rat sera were routinely used to monitor interassay differences.

T_4 and T_3. Serum samples were thawed and levels of total thyroxine (T_4), free T_4, and triiodothyronine (T_3) were measured in duplicate using specific radioimmunoassay (RIA) kits (Diagnostics Products Corporation, Los Angeles, CA). Sensitivity of the total T_4 assay was 5–240 ng/ml; that of the free T_4 assay was 1–100 pg/ml; and that of the T_3 assay was 0.1–6 ng/ml. Because of the surfactant properties of PFOS, a preliminary experiment was conducted to determine whether the chemical might alter performance of the RIA directly. PFOS (at a final concentration of 5 or 10 mg/ml) was added directly to the assay tubes containing the T_4 standards and serum samples from untreated control rats. Under these conditions, PFOS did not interfere with the RIA performance.

TSH and prolactin. Serum samples were thawed, and the levels of TSH and prolactin were quantified by specific RIA. The TSH assay was performed using the following materials supplied by the National Hormone and Pituitary Program (Torrance, CA): iodination preparation NIDDK-rTSH-I-9, reference preparation NIDDK-rTSH-RP-3, and antiserum NIDDK-antirat TSH-RIA-6. Similarly, the prolactin assay was performed with iodination preparation NIDDK-rPRL-I-6, reference preparation NIDDK-rPRL-RP-3, and antiserum NIDDK-antirat PRL-RIA-9. Iodination materials were radiolabeled with ^{125}I (Perkin Elmer/New England Nuclear, Boston, MA) by a modification of the chloramine-T method of Greenwood *et al.* (1963). Labeled TSH or prolactin was separated from the unreacted iodide by gel filtration chromatography, as described previously (Goldman *et al.*, 1986).

Serum was pipetted with the appropriate dilutions to a final assay volume of 500 μl with 100 mM phosphate buffer containing 1% BSA. Reference TSH standards ranging from 0.195 to 200 ng/ml and prolactin standards ranging from 0.39 to 100 ng/ml were prepared by serial dilution. Primary antiserum (200 μl) at a dilution of 1:437,500 prepared in a mixture of 100 mM potassium phosphate, 76.8 mM EDTA, 1% BSA, and 3% normal rabbit serum was then pipetted to each assay tube, vortexed, and incubated at 4°C for 24 h; 100 μl of either ^{125}I-TSH or ^{125}I-prolactin was then added to each tube, vortexed, and incubated for another 24 h. Second antibody (goat antirabbit gamma globulin [Calbiochem, San Diego, CA] at a dilution of 1 U/100 μl) was then added, vortexed, and incubated for a third 24 h. The samples were then centrifuged at 1,260 × *g* for 30 min; the supernatant was aspirated, and the sample tube with pellet was counted in a gamma counter.

Corticosterone. Serum samples were thawed and levels of corticosterone were measured in duplicate using an RIA kit (ICN Biomedical Inc., Costa Mesa, CA). Sensitivity of the assay was 25–1000 ng/ml.

Determination of PFOS Concentrations

Serum samples were diluted and liver samples were homogenized in five volumes of reagent-grade water. An aliquot of each dilution was spiked with the appropriate internal standards. Acetonitrile (5 ml) was added as an extraction solvent, which also served to precipitate the proteins. The samples were shaken at 300 rpm for 20 min and centrifuged at 850 × *g* for 10 min. The supernatant was transferred to a clean tube, diluted with 40 ml of water, and passed through a preconditioned C18 SPE cartridge. PFOS was eluted from the SPE cartridge with 2 ml methanol and analyzed by high-performance liquid

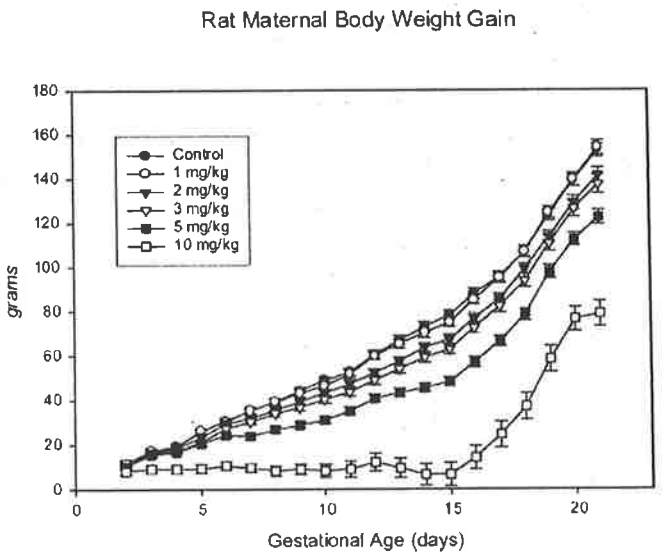

FIG. 1. Effects of PFOS on weight gain in pregnant rats. Each data point represents mean ± SE of determination from 25–50 rats. Two-way ANOVA indicates a significant treatment effect ($p < 0.0001$) and a time × treatment interaction ($p < 0.0001$). Duncan's multiple-range test indicates that, with the exception of the 1 mg/kg group, all dose groups are significantly different from controls. When individual PFOS dose groups are compared with controls, ANOVA indicates a significant treatment effect ($p < 0.0001$) for dose groups at 2 mg/kg and higher. Dunnett's *t*-test indicates significant variations from controls for the 10 mg/kg dose group beginning at GD 4, the 5 mg/kg dose group at GD 5, the 3 mg/kg dose group at GD 7, and the 2 mg/kg dose group from GDs 12 to 17.

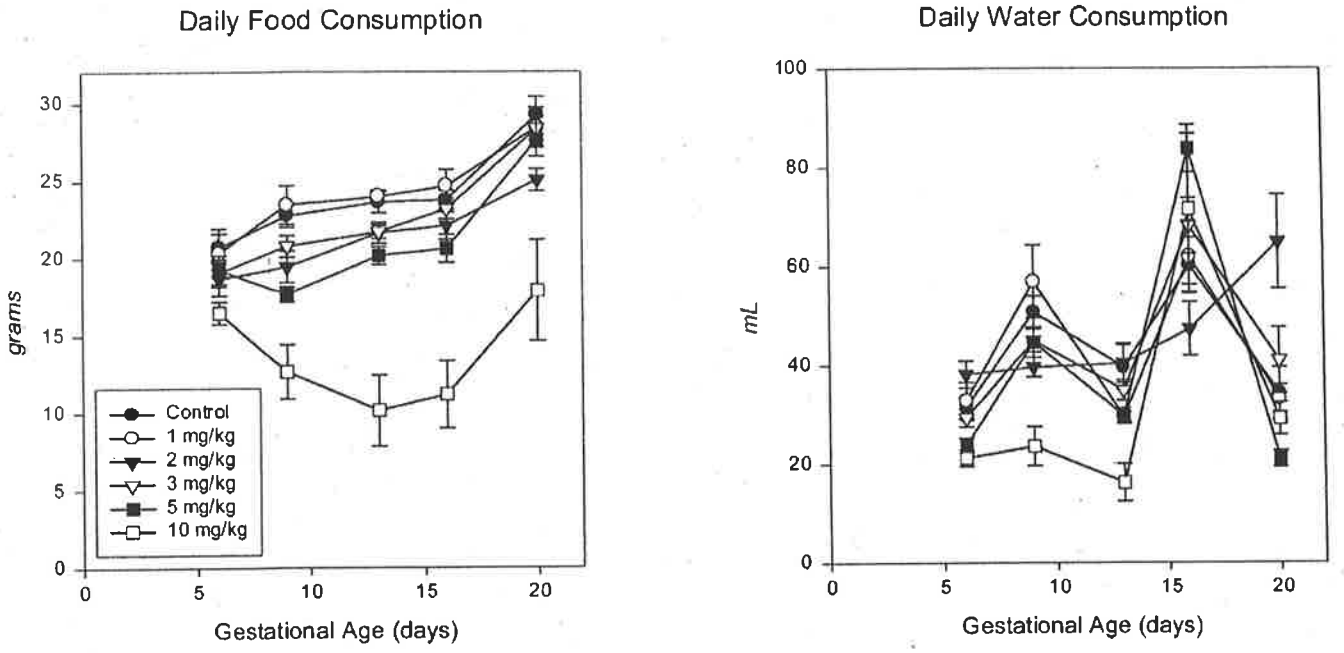

FIG. 2. Effects of PFOS on food and water consumption in pregnant rats. Each data point represents mean ± S.E. of determination from 9–20 rats, with the exception of the 1 and 10 mg/kg dose groups, where $n = 5$. Two-way ANOVA indicates a significant treatment effect ($p < 0.0001$) and a time × treatment interaction ($p < 0.05$) for food consumption. Duncan's multiple-range test indicates that only the 5 and 10 mg/kg dosage groups are significantly different from controls. When individual treatment groups are compared with controls, ANOVA indicates a significant dose effect ($p < 0.0001$) for the 5 and 10 mg/kg dose groups. Dunnett's *t*-test indicates significant variations from controls for the 5 mg/kg dose group from GDs 9 through 16 and for the 10 mg/kg dose group from GD 9 to term. Two-way ANOVA indicates a significant treatment effect ($p < 0.05$) and a time × treatment interaction ($p < 0.0001$) for water consumption. Duncan's multiple-range test indicates that only the 10 mg/kg dose group is significantly different from control values. When individual treatment groups are compared with controls, ANOVA indicates a significant main effect ($p < 0.05$) for the 5 and 10 mg/kg dose groups. Dunnett's *t*-test indicates significant variations at the 0.05 level from control values for the 5 mg/kg dose group on GD 6 and for the 10 mg/kg dose group from GDs 6 to 13.

chromatography-electrospray tandem mass spectrometry (HPLC-ES/MS/MS) according to the method described by Hansen *et al.* (2001).

Data Analysis

Data are presented as means and standard errors. Statistical significance was determined by ANOVA, using individual litter as the statistical unit. Maternal weight gains and food and water consumption were analyzed by ANOVA with repeated measure. When a significant treatment effect or interaction was detected, Duncan's multiple-range test or Dunnett's *t*-test were performed post hoc. Statistically significant differences were determined at $p \leq 0.05$.

The U.S. Environmental Protection Agency (EPA) now uses the benchmark dose (BMD) approach (Barnes *et al.*, 1995; Crump, 1984) for noncancer risk assessment (EPA, 1995). This approach is designed to provide a more quantitative alternative to dose-response assessment than the no-observed-adverse-effect-level (NOAEL) process by constructing mathematical models to fit all data points in the dose-response study and to take data variance into consideration. In this study, BMD_5 and $BMDL_5$ values were calculated for maternal and developmental toxicity after PFOS exposure. BMD_5 refers to the central estimate of the administered dose predicted to cause a 5% increase in response above background, and $BMDL_5$ is defined as the corresponding lower limit of the 95% confidence interval on the BMD (Allen *et al.*, 1994). Benchmark Dose Software (EPA, 2000) was used to calculate the BMD_5 values. Selection of a specific curve-fitting model for the BMD determination was based on the Akaike's Information Criterion (AIC) value. The AIC is equal to $-2L + 2p$, where L is the log-likelihood at the maximum likelihood estimates for the parameters, and p is the number of model parameters estimated. The model that demonstrates "goodness of fit" with the lowest AIC value is presumed to be the most appropriate.

RESULTS

Rat

PFOS reduced maternal weight gain in a dose-dependent manner, significantly in the 2 mg/kg and higher dosage groups (Fig. 1). Dams exposed to 3 mg/kg PFOS showed significant weight deficits ($p < 0.0001$) by GD 7, whereas those exposed to 5 and 10 mg/kg PFOS revealed significant lags ($p < 0.0001$) by GDs 5 and 3, respectively. Effects on maternal weight at the two highest dosage groups were particularly profound. Dams in the 10 mg/kg dosage group failed to gain any weight until the last week of pregnancy. These weight gain deficits corresponded to significant reductions in food and water consumption throughout gestation (Fig. 2).

With the 20-day exposure scheme, PFOS did not affect maternal liver weight in rats (Table 1), but liver/body weight ratio was increased in the 10 mg/kg dosage group, most likely reflecting the marked body weight deficit in these animals. Negligible levels of PFOS were detected in the sera and livers

TABLE 1

Rat Maternal Liver Weight, Serum Chemistry, and Hormones at Term (A); Rat Reproductive Outcome and Fetal Teratology, Examined at Term (B)

	PFOS Exposure level					
	Control	1 mg/kg	2 mg/kg	3 mg/kg	5 mg/kg	10 mg/kg
A. Maternal examinations (*N*)	14	14	9	9	14	16
Liver wt (g)	14.7 ± 0.6^{a}	16.1 ± 0.5^{a}	15.0 ± 0.4^{a}	14.7 ± 0.5^{a}	15.1 ± 0.5^{a}	14.8 ± 0.5^{a}
Relative liver wt (%)	5.0 ± 0.1^{a}	5.2 ± 0.1^{a}	5.1 ± 0.1^{a}	5.0 ± 0.1^{a}	5.3 ± 0.2^{a}	6.0 ± 0.1^{b}
Serum chemistry (*N*)	12	12	9	9	14	12
Cholesterol (mg/dl)	90.6 ± 5.2^{a,b}	98.7 ± 6.3^{a}	92.7 ± 4.6^{a}	98.0 ± 5.1^{a}	88.5 ± 3.1^{a,b}	77.5 ± 4.0^{b}
Triglycerides (mg/dl)	510 ± 44^{a}	522 ± 54^{a}	443 ± 38^{a,b}	421 ± 64^{a,b}	477 ± 34^{a,b}	337 ± 44^{b}
Sorbitol dehydrogenase (units/l)	15.9 ± 2.1^{a}	13.1 ± 2.4^{a}	16.1 ± 1.2^{a}	16.5 ± 2.0^{a}	14.3 ± 2.6^{a}	14.5 ± 3.4^{a}
Serum hormones (*N*)	5	5	—	—	5	5
Corticosterone (ng/ml)	141 ± 22^{a}	147 ± 37^{a}	—	—	102 ± 6^{a}	188 ± 31^{a}
Prolactin (ng/ml)	1.54 ± 0.06^{a}	1.27 ± 0.36^{a}	—	—	0.95 ± 0.15^{a}	1.71 ± 0.39^{a}
B. Fetal examinations (*N*)	13	14	9	8	14	15
Implantation sites (# per dam)	14.7 ± 0.5^{a}	15.7 ± 0.8^{a}	14.3 ± 0.6^{a}	13.8 ± 1.0^{a}	14.8 ± 0.5^{a}	15.2 ± 0.4^{a}
Live fetuses (%)	96.9 ± 1.3^{a}	92.7 ± 2.1^{a}	97.8 ± 1.5^{a}	88.6 ± 8.5^{a}	96.3 ± 1.6^{a}	86.6 ± 6.0^{a}
Body wt (g)	3.87 ± 0.07^{a}	3.85 ± 0.05^{a}	3.74 ± 0.06^{a}	3.86 ± 0.05^{a}	3.73 ± 0.08^{a}	3.38 ± 0.09^{b}
Liver wt (g)	0.29 ± 0.01^{a}	0.32 ± 0.01^{a}	0.31 ± 0.01^{a}	0.30 ± 0.01^{a}	0.28 ± 0.02^{a}	0.28 ± 0.02^{a}
Relative liver wt (%)	7.56 ± 0.24^{a}	8.14 ± 0.24^{a}	8.32 ± 0.16^{a}	7.94 ± 0.34^{a}	7.55 ± 0.28^{a}	7.97 ± 0.22^{a}
Notable skeletal defects (*N*)	13	11	9	7	14	15
Cleft palate (%)	0^{a}	9 ± 9^{a}	14 ± 14^{a}	10 ± 10^{a}	0^{a}	60 ± 13^{b}
Sternal defects (# per fetus)*	1.2 ± 0.3^{a}	1.7 ± 0.3^{a,b}	2.1 ± 0.3^{b,c}	2.6 ± 0.2^{a,b}	2.1 ± 0.2^{a,b}	3.4 ± 0.4^{c}
Ossified proximal phalanges						
(# per forelimb)	1.8 ± 0.4^{a,b,c}	1.9 ± 0.4^{a,b,c}	2.5 ± 0.6^{a,b}	2.0 ± 0.3^{a}	0.8 ± 0.2^{c}	1.0 ± 0.4^{b,c}
(# per hindlimb)	2.1 ± 0.5^{a,b}	2.5 ± 0.5^{a,b,c}	3.9 ± 0.1^{b,c}	3.2 ± 0.3^{c}	1.9 ± 0.5^{a,b}	1.6 ± 0.4^{a}
Notable visceral defects (*N*)	13	6	9	8	14	15
Anasarca (%)	0^{a}	0^{a}	0^{a}	18.1 ± 9.1^{a}	16.6 ± 7.9^{a}	44.3 ± 11.7^{b}
Enlarged right atrium (%)	0^{a}	2.3 ± 2.3^{a}	8.3 ± 8.3^{a,b}	0^{a}	22.9 ± 7.4^{b}	8.7 ± 3.5^{a,b}
Ventricular septal defects (%)	0^{a}	0^{a}	0^{a}	0^{a}	12.9 ± 6.1^{a}	14.6 ± 5.2^{a}

Note. (A) Data represent means ± SE of dams examined as indicated. (B) Data represent means ± S.E. of numbers of litters examined as indicated. Significant differences ($p < 0.05$) were determined by Duncan's multiple-range test and are depicted by different letters (^{a}, ^{b}, and ^{c}); thus, groups sharing the same letter are not significantly different from each other.

*Sternal defects induced by PFOS were primarily bilobed and bipartite.

of the controls (Fig. 3); the source of this slight contamination may have been derived from fish meal in the chow (Seacat *et al.*, 2003). With daily chemical treatment, the serum concentrations of PFOS increased monotonically in proportion to dosage; however, the level of all dosage groups fell toward the end of pregnancy. At term, PFOS concentration as well as the total hepatic burden also increased linearly with PFOS dosage. When these data were expressed as ppm, the liver samples were found to contain approximately four times higher concentrations of PFOS, compared with the corresponding serum samples. Fetal liver weight was not influenced by PFOS exposure (Table 1). An accumulation of PFOS that was proportional to the treatment dosage was also detected in the fetal liver (Fig. 3); based on concentration, fetal livers appeared to contain approximately half as much PFOS as their maternal counterparts.

Analysis of the serum chemistry of pregnant rats at term revealed that PFOS caused a significant reduction in circulating cholesterol and triglycerides only in the 10 mg/kg dosage group (Table 1). Sorbitol dehydrogenase, glucose, bile acid, and bilirubin levels were not altered by PFOS treatment (data not shown). In contrast, PFOS produced a marked reduction in both total and free serum T_4 in all dosage groups as early as GD 7 (Fig. 4) and in serum T_3 to a lesser extent, as well. However, no difference in serum TSH was observed among the treatment groups. An additional study was conducted with adult female (nonpregnant) rats in which the animals were exposed to PFOS (3 or 5 mg/kg) for 20 days. Similar to findings in pregnant rats, serum T_4 (both total and free) and T_3 levels in the nonpregnant rats were markedly reduced by the chemical treatment (as early as 3 days after the initiation of exposure) (Fig. 5). The pattern of TSH response is somewhat confounding and appears to be dose dependent. For the 3 mg/kg dosage group, a significant elevation (47%) of serum TSH was detected after 7 days of PFOS treatment. This hormonal increase was maintained for another week, although it was no longer statistically different from controls. After 20 days of chemical treatment, the alteration of TSH was completely attenuated. In contrast, the serum TSH levels in the 5 mg/kg dosage group were

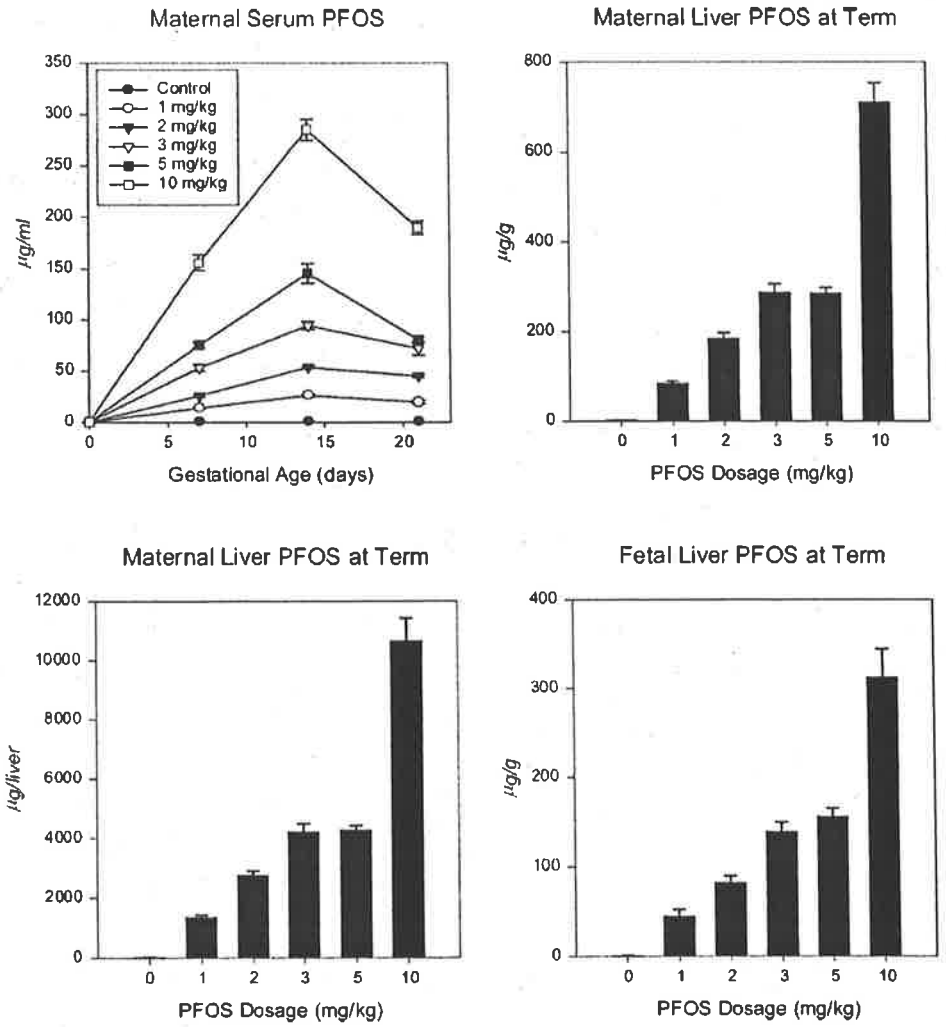

FIG. 3. Concentrations of PFOS in rat maternal serum throughout gestation and maternal and fetal liver at term. Each data point or bar represents mean ± SE of determination from 9–14 rats.

slightly lower than controls at the initial stages of PFOS exposure (by 26% and 21%, respectively, after 3 and 7 days); these changes were also abolished after 20 days of treatment. By comparison, PFOS did not alter serum corticosterone or prolactin levels appreciably in the pregnant rats at term (Table 1).

In utero exposure to PFOS throughout gestation did not produce adverse effects on the number of live fetuses or postimplantation loss in the treated dams (Table 1). However, a significant reduction of fetal weight was apparent in the 10 mg/kg group. Gross and skeletal examinations revealed a significant increase in the incidence of cleft palate, defective sternebrae, anasarca, enlarged right atrium, and ventricular septal defects, primarily in the fetuses exposed to the highest level of PFOS (Table 1).

Mouse

PFOS-induced deficits in maternal weight gain were not as pronounced in the mouse as in the rat. Statistically significant differences in body weight gain were observed only in the 20 mg/kg dosage group at late gestation (Fig. 6). Likewise, food and water consumption were less affected by the chemical exposure (Fig. 7). In contrast, PFOS treatment increased maternal liver weight in a dose-dependent fashion; indeed, in the highest dosage group (20 mg/kg), the livers almost doubled their weight, compared with those in controls (Table 2). Serum PFOS concentrations in the mouse were comparable with those found in the rat (Fig. 8); for the 10 mg/kg dosage group, the mean (± SE) maternal rat serum at term was 190 ± 7 µg/ml, and the maternal mouse serum at term was 179 ± 35 µg/ml. Additionally, serum PFOS in the mouse appeared to reach a saturated concentration at 250 µg/ml. A similar pattern of PFOS accumulation and saturation was seen in the maternal mouse liver. Indeed, in the 10 mg/kg dosage group, the rat maternal liver PFOS concentration was 710 ± 44 µg/g, and that for the mouse liver was 560 ± 52 µg/g.

As observed in the rat, maternal serum triglycerides were

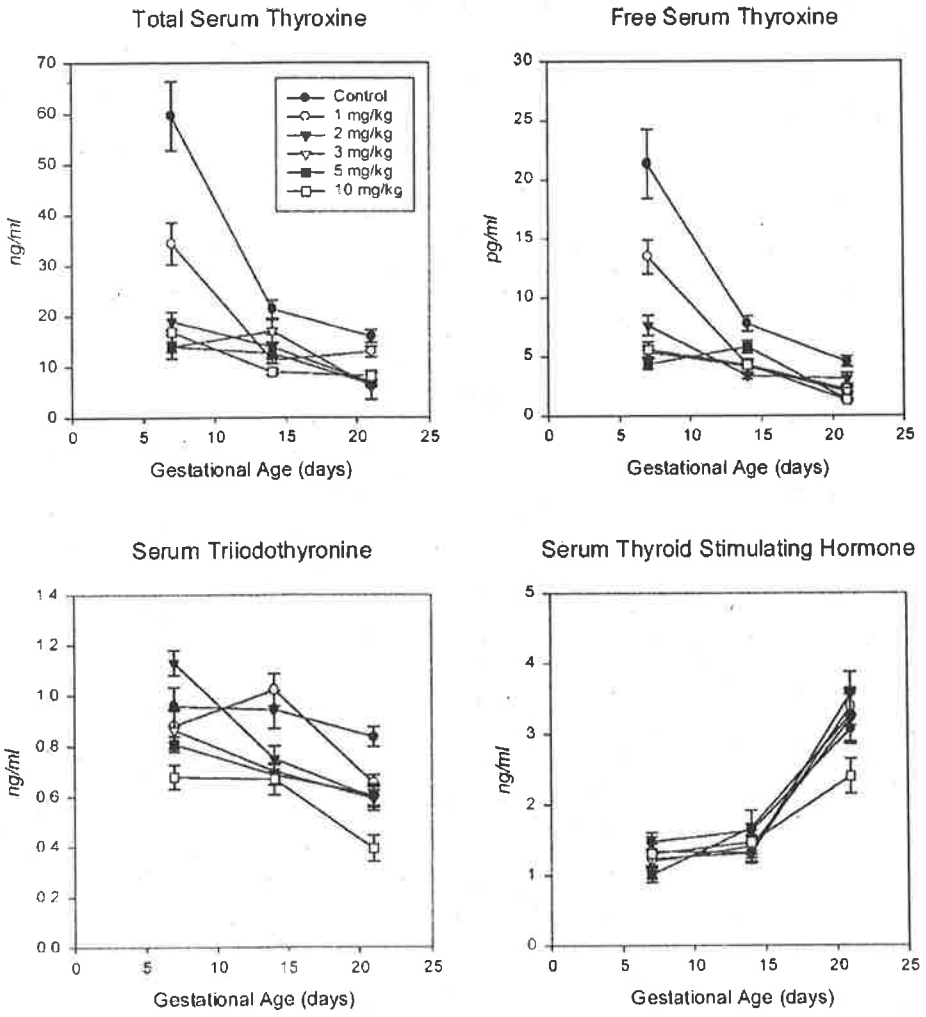

FIG. 4. Effects of PFOS on circulating thyroid hormones in pregnant rats. Each data point represents mean ± SE of duplicate determination from 9–14 rats. Two-way ANOVA indicates significant effects on time and treatment, and time × treatment interaction for serum total and free T_4 levels ($p < 0.0001$) and for serum T_3 levels ($p < 0.002$). Duncan's multiple-range test indicates that all doses are significantly different from control values at all time points evaluated for T_4; whereas significant differences from controls are detected in 10 mg/kg group on GD 7, in 3, 5, and 10 mg/kg groups on GD14, and all doses groups on GD 21 for T_3. For serum TSH, two-way ANOVA indicates a significant effect of time ($p < 0.0001$) but not of treatment, and no interaction.

also significantly lowered by PFOS in the mouse in a dose-dependent manner (Table 2), although neither serum cholesterol nor sorbitol dehydrogenase was significantly altered. As seen in the rat, a rapid decline of serum thyroxine was noted in the mouse during pregnancy (Fig. 9). However, the adverse effect of PFOS on thyroid hormones was less pronounced in the mouse than in the rat. Serum T_4 levels were reduced by the chemical treatment in a dose-dependent manner by GD 6, but hormone levels in the PFOS-exposed mice were no longer different from controls during the last week of pregnancy (Fig. 9).

Exposure of pregnant mice to PFOS throughout gestation did not alter the number of implantation sites; however, a significant increase in postimplantation loss was seen in the 20 mg/kg dosage group (Table 2). Small but significant reductions of fetal weight were detectable in the 10 and 15 mg/kg dosage groups. In addition, fetal liver weights (absolute and relative) were significantly elevated at 20 mg/kg. Fetal examination revealed cleft palate, defective sternebrae, enlargement of the right atrium, and ventricular septal defects, but primarily in the higher dosage groups (15 and 20 mg/kg) (Table 2).

DISCUSSION

Consistent with recent findings with Cynomolgus monkeys (Seacat *et al.*, 2002), accumulated body burdens of PFOS in pregnant rodents were found to be directly proportional to exposure levels in this study. At term, the correlation coefficients (r^2) between administered dosage and PFOS levels in the rat were 0.980 for serum and 0.964 for liver. In the mouse, saturation kinetic was apparent between 15 and 20 mg/kg; hence, for dosages below 15 mg/kg, the r^2 between treatment dosages and PFOS levels was 0.993 for serum and 0.989 for liver. The existence of such linear relationships across these three species and a wide dose range (0.03 mg/kg/day for monkey to 15 mg/kg/day for mouse) lends support to similar crude extrapolations for other species and exposure levels. PFOS was preferentially accumulated in the liver; the ratio of

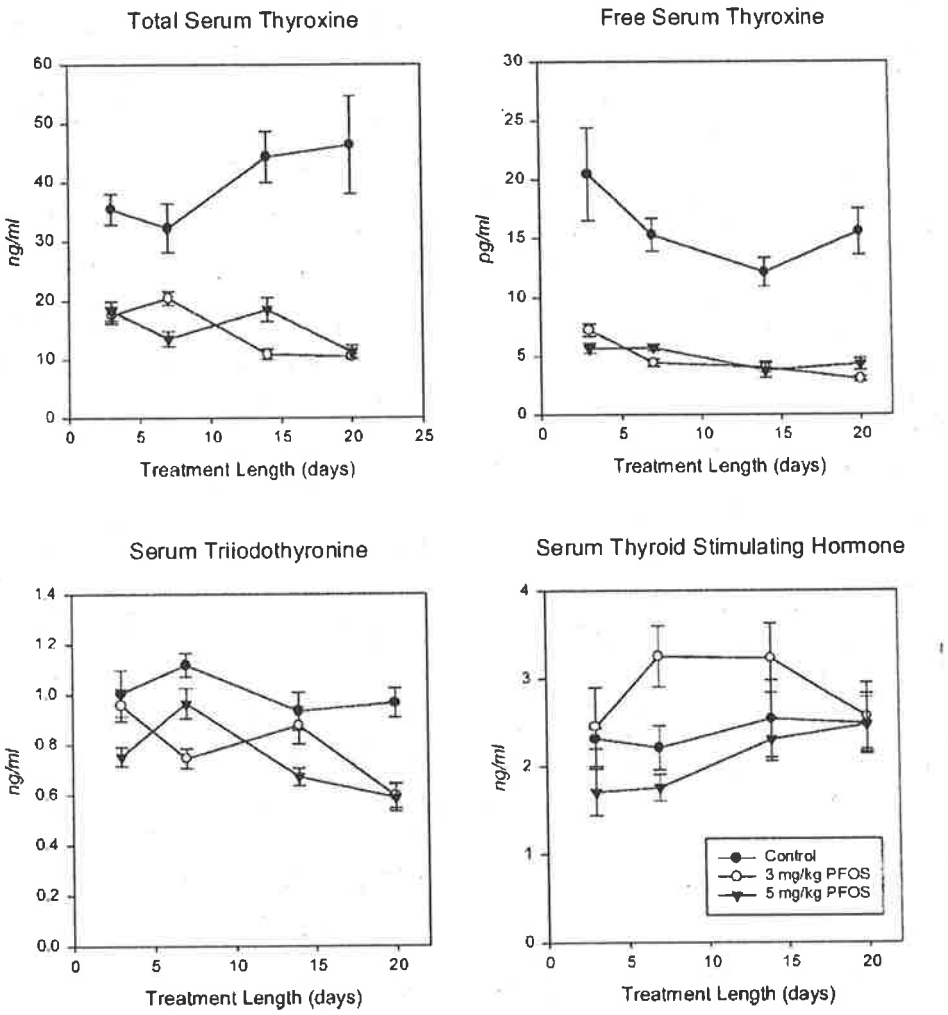

FIG. 5. Effects of PFOS on circulating thyroid hormones in adult female nonpregnant rats. Each data point represents mean ± SE of duplicate determination from 6–8 rats. Two-way ANOVA indicates a significant treatment effect ($p < 0.001$) and a time × treatment interaction ($p < 0.02$) for total T_4; a significant treatment effect ($p < 0.001$) but no interaction for free T_4; and a significant treatment effect ($p < 0.005$) and a time × treatment interaction ($p < 0.005$) for T_3. When individual PFOS dose groups are compared with controls, ANOVA indicates a significant treatment effect ($p < 0.0001$) for both 3 and 5 mg/kg dose groups. For TSH, two-way ANOVA indicates a significant treatment effect ($p < 0.004$) but no interaction; Duncan's multiple-range test indicates a significant difference between the 3 mg/kg dose group and controls and between the 3 mg/kg and 5 mg/kg dose groups but not between the 5 mg/kg dose group and controls.

serum to liver concentration was approximately 1:4 in both rodent species, regardless of the administered dosages and comparable with the reported values for rat (at approximately 1:5, Seacat *et al.*, 2003) and monkey (at approximately 1:2, Seacat *et al.*, 2002). These data are consistent with the previous observation of enterohepatic circulation of PFOS (Johnson *et al.*, 1984). PFOS levels in the fetal liver were nearly half of those in the maternal counterparts, regardless of administered dose. Although the PFOS levels in fetal circulation were not measured in this study, data from a postnatal evaluation of PFOS toxicity described in the companion article (Lau *et al.*, 2003) indicate that serum concentrations of the fluorochemical in the newborns were comparable to those in maternal circulation. Thus, the lower accumulation of hepatic PFOS in the fetuses would likely suggest a reduced capacity of chemical uptake/storage in the liver or immaturity of the enterohepatic circulation. However, it must be cautioned that the pharmacokinetic properties of PFOS, particularly during pregnancy, are complex and have not yet been characterized. For instance, the decline of serum PFOS levels in the rat at term (Fig. 3) most likely reflects a marked expansion of maternal blood volume that is characteristic of the late term of pregnancy (Barron, 1987; Tam and Chan, 1977). Hence, an accurate profile of PFOS disposition, particularly during pregnancy, must await the construction of a detailed pharmacokinetic model for the fluorochemical.

Maternal toxicity of PFOS, indicated by deficits in weight gain during pregnancy, was observed in both rat and mouse. In both rodents, the severity of the adverse effects was dose-dependent; at term, a BMD_5 for maternal weight reduction is estimated at 0.22 mg/kg and a $BMDL_5$ at 0.15 mg/kg for the rat, and a BMD_5 of 15.2 mg/kg and a $BMDL_5$ of 3.1 mg/kg are determined for the mouse (polynomial model). In the rat, the lag in weight gain during pregnancy was particularly pronounced in the two highest dosage groups (5 and 10 mg/kg), which exhibited marked reductions of food and water intake. The PFOS-induced reductions of maternal weight gain in the rat and mouse seen here are comparable to similar alterations produced by the fluorochemical in the rabbit or by N-alkyl perfluorooctanesulfonamido ethyl alcohol in the rat and rabbit

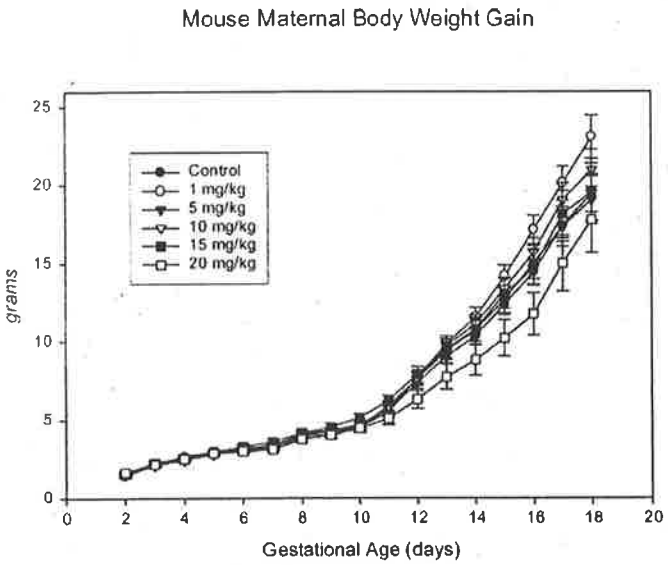

FIG. 6. Effects of PFOS on body weight gain in pregnant mice. Each data point represents mean ± SE of determination from 60–80 mice. Two-way ANOVA indicates a significant treatment effect ($p < 0.0001$) and a time × treatment interaction ($p < 0.02$). Duncan's multiple-range test indicates that the 10 and 20 mg/kg dose groups vary significantly from control values and from each other.

(Case *et al.*, 2001), indicating that the adverse effect on maternal weight gain may be a common feature of toxicity for the perfluorochemicals. Liver enlargement with associated histological abnormalities is another feature often seen after exposure to PFOS and related compounds (Case *et al.*, 2001; Haughom and Spydevold, 1992; Ikeda *et al.*, 1987; Seacat *et al.*, 2002; 2003; Sohlenius *et al.*, 1993). A somewhat similar finding was obtained in our studies. An increase of liver weight is generally observed in rodents during pregnancy (by about 24% in rat, Buelke-Sam *et al.*, 1982; and 56% in mouse, observation in our laboratory, data not shown). Above and beyond this physiological change, significant elevations of hepatic weight were found in the PFOS-exposed mice, and increases in the high-dose groups were as much as twofold over the corresponding controls. Serum triglycerides in these mice were significantly reduced. Interestingly, a comparable PFOS-induced liver enlargement was absent in the pregnant rat; the small increase in the relative liver weight in the 10 mg/kg dosage group largely reflected the reduction of body weight, rather than a net increase of liver weight. Serum cholesterol and triglycerides in the rat were also not altered appreciably by PFOS exposure. An explanation for these disparate findings (compared to the results reported by Seacat *et al.*, 2003, for instance) is not readily available and may be attributed to the relatively short duration of PFOS exposure (20 days) in our study (compared to 14 weeks in the Seacat study). Nonetheless, the high sensitivity to PFOS-induced liver toxicity in the mouse should be noted, with a BMD_5 and a $BMDL_5$ of liver weight increase estimated at 2.61 mg/kg and 1.31 mg/kg (Hill model), respectively.

Seacat and co-workers (2002) reported reductions of serum T_3 and elevations of TSH in monkeys after exposure to PFOS for 182 days. In this study, PFOS produced a much more marked reduction of both T_3 and T_4 in pregnant rats and at a much earlier onset. For the T_4 effects at GD 7, a BMD_5 at 0.23 mg/kg and a $BMDL_5$ at 0.05 mg/kg (Hill model) are estimated. Interestingly, the accumulated serum PFOS level where thyroid imbalance was detected in the monkey (171 ppm) is comparable to that in the pregnant rat (53–155 ppm). Yet in rats, despite these deficits in circulating hormones, a feedback elevation of TSH through activation of the hypothalamic-pituitary-thyroid (HPT) axis was not apparent. Because the level of serum T_4 (and to a lesser extent, T_3) falls and that of TSH rises during pregnancy (Versloot *et al.*, 1994; Figure 4 in this study), these physiological changes might have masked the true effects of PFOS. However, the T_3 and T_4 results from the study with nonpregnant female rats by and large substantiated the findings in pregnant dams, discounting potential confounding effects of pregnancy. The dose-dependent, paradoxical

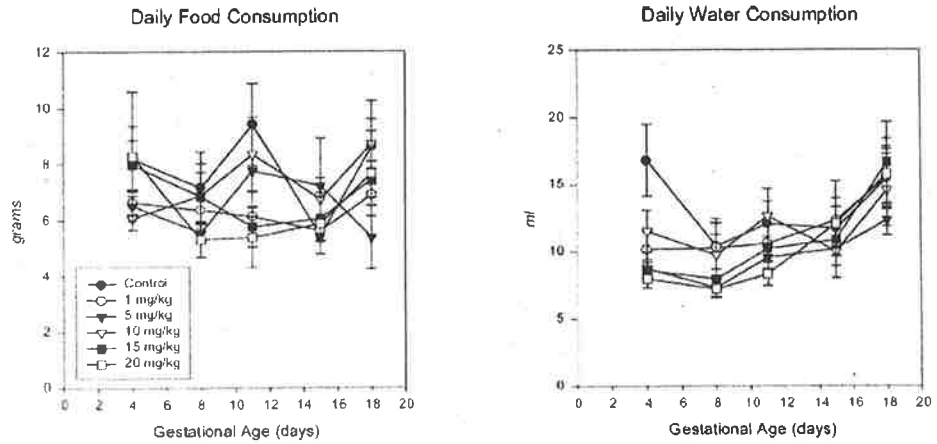

FIG. 7. Effects of various doses of PFOS on food and water consumption in pregnant mice. Each data point represents mean ± SE of determination from 14–19 mice. Two-way ANOVA indicates no significant treatment effect or interaction associated with food consumption but a significant treatment effect ($p < 0.05$) for water consumption.

TABLE 2

Mouse Maternal Liver Weight and Serum Chemistry at Term (A), and Mouse Reproductive Outcome and Fetal Teratology, Examined at Term (B)

	PFOS Exposure level					
	Control	1 mg/kg	5 mg/kg	10 mg/kg	15 mg/kg	20 mg/kg
A. Maternal examinations (*N*)	20	27	29	24	26	25
Liver wt (g)	2.19 ± 0.08^{a}	$2.46 \pm 0.10^{a,b}$	2.78 ± 0.08^{b}	3.86 ± 0.10^{c}	4.61 ± 0.17^{d}	4.25 ± 0.21^{c}
Relative liver wt (%)	7.42 ± 0.24^{a}	7.36 ± 0.20^{a}	9.05 ± 0.28^{b}	12.25 ± 0.19^{c}	14.45 ± 0.34^{d}	15.51 ± 0.26^{e}
Serum chemistry (*N*)	19	19	20	21	9	5
Cholesterol (mg/dl)	$68.9 \pm 4.9^{a,b}$	$69.7 \pm 6.2^{a,b}$	83.3 ± 6.0^{a}	83.1 ± 5.4^{a}	$78.0 \pm 5.8^{a,b}$	58.4 ± 10.2^{b}
Triglycerides (mg/dl)	284 ± 18^{a}	$220 \pm 21^{a,b}$	196 ± 18^{b}	$162 \pm 11^{b,c}$	$191 \pm 19^{b,c}$	119 ± 18^{c}
Sorbitol dehydrogenase (units/l)	20.3 ± 3.1^{a}	23.5 ± 3.6^{a}	22.3 ± 4.8^{a}	23.5 ± 3.3^{a}	20.7 ± 3.1^{a}	23.3 ± 6.1^{a}
B. Fetal examinations (*N*)	19	26	12	24	18	11
Implantation sites (# per dam)	12.3 ± 0.9^{a}	13.7 ± 1.1^{a}	11.1 ± 1.1^{a}	12.7 ± 0.8^{a}	12.4 ± 1.4^{a}	12.9 ± 0.6^{a}
Live fetuses (%)	97.9 ± 1.0^{a}	96.6 ± 1.1^{a}	$91.9 \pm 3.1^{a,b}$	96.0 ± 0.9^{a}	$95.3 \pm 1.0^{a,b}$	89.1 ± 5.5^{b}
Body wt (g)	1.05 ± 0.03^{a}	1.05 ± 0.01^{a}	1.05 ± 0.04^{a}	$0.97 \pm 0.01^{b,c}$	0.94 ± 0.02^{b}	$1.03 \pm 0.02^{a,c}$
Liver wt (mg)	$77 \pm 2^{a,b,c}$	67 ± 4^{a}	$88 \pm 5^{c,d}$	$76 \pm 2^{a,b}$	$79 \pm 3^{b,c}$	91 ± 3^{d}
Relative liver wt (%)	7.13 ± 0.20^{a}	6.26 ± 0.39^{b}	7.93 ± 0.20^{a}	7.99 ± 0.18^{a}	7.95 ± 0.16^{a}	9.07 ± 0.17^{c}
Notable skeletal defects (*N*)	8	3	4	9	11	6
Cleft palate (%)	0^{a}	0^{a}	0^{a}	$1.8 \pm 1.8^{a,b}$	21.1 ± 8.3^{b}	73.2 ± 11.7^{c}
Sternal defects (# per fetus)*	0.5 ± 0.2^{a}	$1.0 \pm 0.3^{a,b}$	$2.1 \pm 0.4^{b,c}$	$2.1 \pm 0.2^{b,c}$	$2.6 \pm 0.2^{b,c,d}$	3.3 ± 0.7^{d}
Notable visceral defects (*N*)	9	5	3	8	12	8
Enlarged right atrium (%)	0^{a}	0^{a}	0^{a}	25.8 ± 7.8^{b}	22.5 ± 7.2^{b}	34.5 ± 9.2^{b}
Ventricular septal defects (%)	1.9 ± 1.9^{a}	0^{a}	0^{a}	5.4 ± 2.7^{a}	4.9 ± 3.3^{a}	30.0 ± 15.6^{b}

Note. (A) Data represent means ± SE of dams examined as indicated. (B) Data represent means ± SE of litters examined as indicated. Significant differences ($p < 0.05$) were determined by Duncan's multiple-range test and are depicted by different letters (^{a}, ^{b}, ^{c}, ^{d}, and ^{e}); thus, groups sharing the same letter are not significantly different from each other.

*Sternal defects induced by PFOS were primarily bilobed and bipartite.

responses of serum TSH in the nonpregnant rats are intriguing. The near-identical response patterns between total and free T_4 rule out the potential involvement of the hormone binding proteins. Although feedback increases of the pituitary hormone in the 3 mg/kg dosage group were relatively small (27–50%), compared with the two- to threefold increase induced by propylthiouracil (Cooper *et al.*, 1983), these changes nonetheless indicate the integrity of the HPT axis. The recovery of TSH after a transient response, despite the persistent reductions of serum T_3 and T_4, suggests that the homeostatic balance of thyroid hormone economy may have been reset. These findings resemble those previously reported with long-term chemical

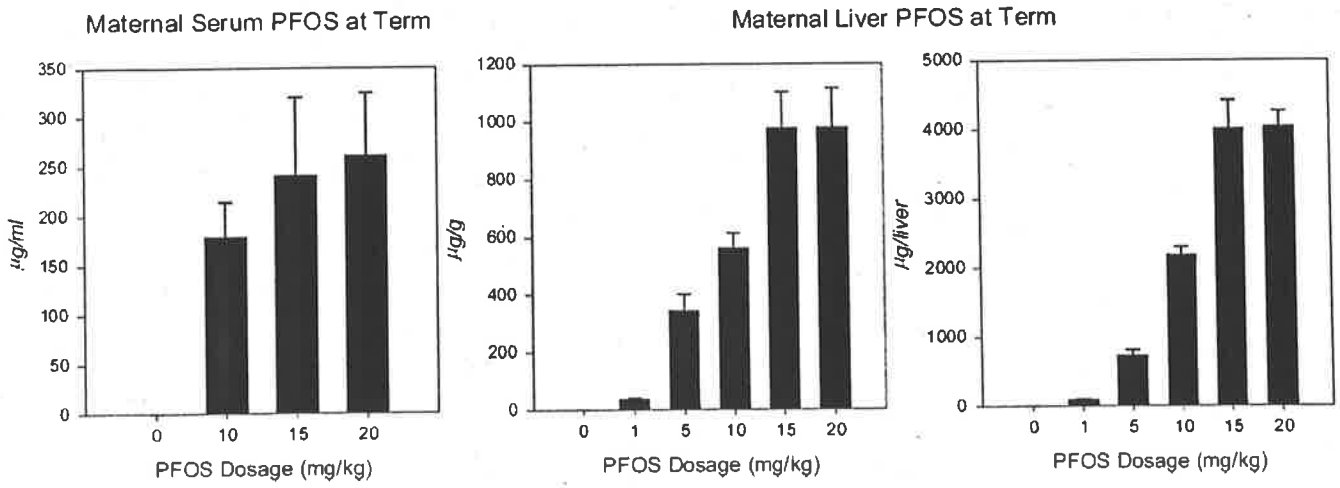

FIG. 8. Concentrations of PFOS in mouse maternal serum and liver at term. Each data point or bar represents mean ± SE of determination from six mice.

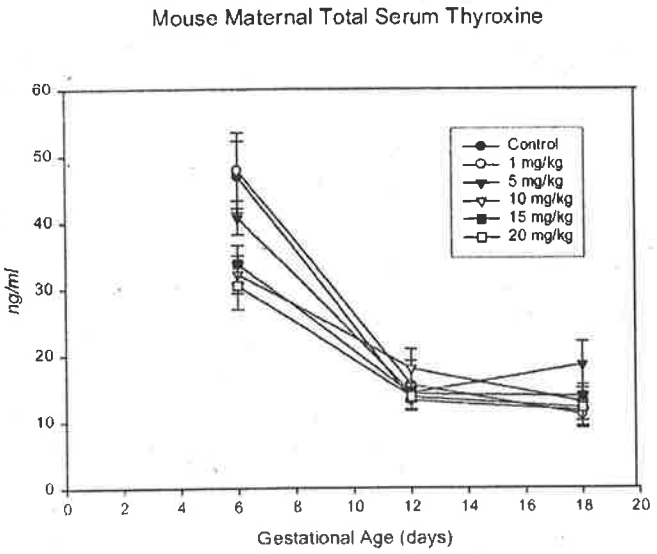

FIG. 9. Effects of PFOS on circulating total T_4 levels in pregnant mice. Two-way ANOVA indicates a significant effect of time ($p < 0.0001$) and a time × treatment interaction ($p < 0.005$). Dunnett's *t*-test demonstrates that the T_4 level associated with the 20 mg/kg dose group is significantly different at the 0.05 level from the control value on GD 6.

disruption of the thyroid axis (Biegel *et al.*, 1995). The absence of serum TSH elevation (through the HPT feedback mechanism) in the 5 mg/kg dosage group is perplexing. In fact, the TSH levels of these rats were slightly depressed in the initial stage of PFOS exposure. These results point toward a more complex, dose-dependent effect of the fluorochemical that is not yet fully understood.

Pregnant mice exhibited a pattern of PFOS-induced T_4 reductions similar to that seen in the pregnant rat, with a BMD_5 of 0.51 mg/kg and a $BMDL_5$ of 0.35 mg/kg estimated at GD 6 (linear model). This profile of thyroid hormone imbalance (reductions of T_3 and T_4 without a compensatory elevation of TSH) produced by PFOS, though puzzling, is not unique. Chemically induced decreases of serum T_4 and T_3 without significant feedback increase of TSH have been reported with polychlorinated biphenyls (PCBs) (Goldey *et al.*, 1995; Liu *et al.*, 1995). The mechanism(s) underlying the thyroidal effects of PFOS remains to be elaborated. Similar patterns of alterations between total and free T_4 rule out involvement of hormone binding proteins. Alternatively, the hepatotoxicity of PFOS presents a prime possibility. Altered thyroid hormone metabolism through induction of hepatic enzymes has been described with chemicals such as phenobarbital, pregnenolone-16α-carbonitrile, 3-methylcholanthrane, PCB, and brominated diphenyl ethers (Byrne *et al.*, 1987; Liu *et al.*, 1995; Zhou *et al.*, 2002). Regardless, the thyroid hormone deficits produced by PFOS during pregnancy are of potential concern, particularly if the feedback mechanism via the HPT axis is compromised. Thyroid hormones play a critical role in the normal development of the lung, inner ears, and nervous system (particularly CNS), and they regulate growth, metabolic rate, cardiac performance, and body temperature (Lucas *et al.*, 1988; Glinoer, 2001). During *in utero* development, the embryo and fetus rely completely on maternal supplies of thyroid hormones through placental transfer until maturation of the fetal thyroid gland toward late gestation. Perinatal hypothyroidism has been shown to cause retardation of neurodevelopment and stunted growth (Porterfield, 1993), and recent epidemiological findings indicate that even subtle changes of the thyroid economy (subclinical hypothyroidism) during maturation may have long-lasting effects on the development of intellectual and motor skills (Haddow *et al.*, 1999). Decreased availability of maternal T_4 to the developing brain poses an increased risk of poor neuropsychological development, and a direct relationship between the degree of neonatal hypothyroxinemia and subsequent neurodevelopment has been established (Morreale de Escobar *et al.*, 2000).

In view of the profound deficits in maternal weight gain in PFOS-exposed rats, it was surprising to find little adverse effect on the viability of the fetuses at term. In fact, only small decrements of fetal weight were noted. Similar results were obtained with the mouse, even at higher exposures. On the other hand, anasarca, craniofacial malformation (cleft palate), cardiac defects (ventricular septal defects, enlargement of the right atrium), and delayed ossification (sternebrae, phalanges) were detected in the PFOS-exposed fetuses. A BMD_5 for the sternal defects is estimated at 0.31 mg/kg, with a $BMDL_5$ at 0.12 mg/kg (logistic model); and a BMD_5 for cleft palate at 8.85 mg/kg, with a $BMDL_5$ at 3.33 mg/kg (logistic model). The enlarged right atrium may be associated with complications of pulmonary function. The mouse essentially produced an identical teratological profile. For comparison, BMD_5 and $BMDL_5$ for the sternal defects in the mouse are estimated at 0.06 mg/kg and 0.02 mg/kg, respectively (logistic model); those for cleft palate are 7.03 mg/kg and 3.53 mg/kg, respectively (NCTR model). These results are in agreement with previous teratological findings with lithium perfluorooctane sulfonate and N-ethylperfluorooctanesulfonamido ethyl alcohol in the rat (Case *et al.*, 2001; Henwood *et al.*, 1994) and PFOS in the

TABLE 3
Species Comparison of the Benchmark Doses for Various Parameters of PFOS Maternal and Developmental Toxicity

	Rat		Mouse	
	BMD_5 (mg/kg)	$BMDL_5$ (mg/kg)	BMD_5 (mg/kg)	$BMDL_5$ (mg/kg)
Maternal body wt at term	0.224	0.150	15.15	3.14
Maternal serum total T_4 (Rat GD 7; mouse GD 6)	0.234	0.046	0.513	0.352
Maternal liver wt at term	—	—	2.61	1.31
Fetal sternal defects	0.313	0.122	0.055	0.016
Fetal cleft palate	.85	3.33	7.03	3.53

rabbit (Case *et al.*, 2001). Nonetheless, it should be noted that a preponderance of these structural abnormalities was found in the highest PFOS dosage group (10 mg/kg for the rat and 20 mg/kg in the mouse). Although a significant reduction of weight gain and food consumption was noted in this group of pregnant rats, malnutrition is not likely the sole factor accounting for the induction of birth defects. Indeed, equivalent or higher incidence of malformations was seen in the mouse fetuses, yet the deficits of weight gain and food consumption in the mouse dams were much less extensive than those of the rat.

Previous studies have shown that PFOS can interfere with cholesterol synthesis through inhibition of HMG CoA reductase activity (Haughom and Spydevold, 1992). Because cholesterol is known to play a role in development through the molecular signaling of *sonic hedgehog* (Brewer *et al.*, 1993), alterations of this metabolic precursor may be involved in the mechanism of dysmorphogenesis (Fitzky *et al.*, 2001). Yet in this study, maternal serum cholesterol was not significantly lowered by PFOS treatment in either rodent species. Indeed, results from a preliminary study (Luebker *et al.*, 2002b) indicated that cholesterol or mevalonic acid supplement failed to ameliorate PFOS-induced developmental toxicity in the rat. Alternatively, altered thyroid status in the dam may raise concerns regarding developmental toxicity. Thyroid hormone effects on cell proliferation and differentiation, as well as on organ growth and maturation, have been well documented. On the other hand, changes of these parameters are often subtle (for instance, at a functional rather than morphological level) and not easily discernible by standard teratological assessment. Hence, evaluations for potential developmental toxicity of PFOS have been extended to postnatal examination, and the results are described in a companion article (Lau *et al.*, 2003).

In summary, exposure to PFOS during pregnancy led to significant physiological alterations in the rat and mouse that are indicative of maternal toxicity, as well as to anatomical defects observed in the fetuses at term at high dosages. These adverse outcomes are dose-dependent and can be correlated with body burden of the fluorochemical. Generally, the mouse appeared to be a less sensitive species than the rat in regard to the PFOS-induced toxicity. A species comparison of the benchmark doses for various parameters is provided in Table 3.

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DOCUMENT 47

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Deterimination of Serum Elimination Half-Lives of Ammonium Perfluorooctanoate, Perfluorooctane Sulfonic Acid, Perfluoroheaxane sulfonic acid and Total Organic Fluorine in Decatur Chemical Plant Retirees

The objective of this study is quantitate the human serum elimination half life of perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA) , perfluoroohexanesulfonate (PFOS) and total organic fluorine (TOF). Blood will be collected biannually from approximately 25 retireed 3M Decatur employees for a period of 5 years. Upon collection and analysis of the third sample (baseline, 6 months and then 12 months) and thereafter, an interim draft half-life analysis report will be prepared.

PROTOCOL

Epidemiology,220-3W-05
Medical Department
3M Company
St. Paul, MN 55144

Date: October 14, 1998

Title: DETERMINATION OF SERUM ELIMINATION HALF-LIVES OF AMMONIUM PERFLUOROOCTANOATE, PERFLUOROOCTANE SULFONIC ACID, PERFLUOROHEXANE SULFONIC ACID AND TOTAL ORGANIC FLUORINE IN 3M DECATUR CHEMICAL PLANT RETIREES.

Study Start Date:	Protocol Number # 0007 IRB Approval # 98095 Exempt XX Expedited
Estimated Date of Final Report:	June, 2004
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Principal Investigator:	Jean M. Burris RN, MPH
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ABSTRACT

3M manufactures products which contain chemical compounds, either as intentional components or residual impurities, that have as a parent molecule, perfluorooctane sulfonyl fluoride (POSF). These compounds may be expected to transform metabolically, to an undetermined degree, to PFOS as an end-stage metabolite. Other compounds manufactured by 3M include ammonium perfluorooctanoate (PFOA) and perfluorohexane sulfonic acid (PFHS). These molecules enter a number of product applications (e.g., surfactants, food packaging additives, polymers).

The biological elimination rates of fluorochemicals have been studied in rats, pregnant rats, female rabbits and hamsters. Biological half-lives have been calculated from the studies in rats and dogs. In addition, the half-life of serum total organic fluorine in a fluorochemical worker was estimated using serial collections of serum total organic fluorine. These studies reveal that PFOS and PFOA may persist in the body for prolonged periods, and with continuing exposure, accumulate over time in biological systems. To date, the actual elimination half-life of perfluorooctane sulfonate (PFOS), ammonium perfluorooctanoate (PFOA), perfluorohexane sulfonic acid (PFHS), and total organic fluorine (TOF) in human serum is not completely understood. The objectives of this study are to quantitate the elimination half life in human serum of PFOA, PFOS, PFHS, and TOF. These half -lives will be calculated from declines in serum fluorochemical levels in 3M Decatur Chemical Plant Retirees.

INTRODUCTION

3M manufactures products which contain chemical compounds, either as intentional components or residual impurities, that have as a parent molecule, perfluorooctane sulfonyl fluoride (POSF). These chemicals include: perfluorooctane sulfonate (PFOS), N-ethyl perfluorooctanesulfonamide, N-ethyl perfluorooctanesulfonamido ethanol, N-methyl perfluorooctanesulfonamido ethanol and chemicals derived from it, and the mixture of mono-, di- and tri [N-ethyl perfluorooctane sulfonamidoethyl] phosphates. There may be other precursors in the workplace, including perfluorohexanesulfonyl fluoride (PHSF). These molecules enter a number of product applications (e.g., surfactants, food packaging additives, polymers). These compounds may be expected to transform metabolically, to an undetermined degree, to PFOS (and PFHS) as an end-stage metabolite. Potassium perfluorooctane sulfonate ($C_8F_{17}OSO_2K^+$) is itself, a surfactant used as a wetting and foaming agent in industrial and commercial processes. (Olsen, et al., 1998)

Subchronic studies in rats and primates suggest there may be a potential for cumulative toxicity with PFOS over time with the primary effect related to metabolic wasting. These molecules have long elimination half lives in biological systems. Although the mechanism of toxicity is not fully understood, toxicity may be due to an effect on peroxisome proliferation, fatty acid metabolism, membrane function, protein synthesis and/or mitochondrial bioenergetics. (Olsen, et al., 1998)

The biological elimination rates of fluorochemicals have been studied in rats, pregnant rats, female rabbits and hamsters. Biological half-lives have been calculated from the studies in rats and dogs. In addition, the half-life of serum total organic fluorine in a fluorochemical worker

was estimated using serial collections of serum total organic fluorine. These studies reveal that PFOS and PFOA may persist in the body for prolonged periods, and with continuing exposure, accumulate over time in biological systems. To date, the actual elimination half-life of perfluorooctane sulfonate (PFOS), ammonium perfluorooctanoate (PFOA), perfluorohexane sulfonic acid (PFHS), and total organic fluorine (TOF) in human serum is not completely understood.

The objectives of this study are to quantitate the elimination half life in human serum of PFOA, PFOS, PFHS, and TOF; these half lives will be calculated from declines in serum fluorochemical levels in 3M Decatur Chemical Plant Retirees. This information is necessary in order to: 1) plan the most appropriate periodicity of medical surveillance and 2) guide medical decision making of workers from fluorochemical production facilities when those workers' serum fluorochemical levels are determined to be unsafely elevated. A benefit of this research will be to understand the natural history of fluorochemical excretion.

LITERATURE REVIEW

AMMONIUM PERFLUOROOCTANOATE (PFOA or FC-143)

Excretion rates of PFOA have been observed in rats, and found to be different by gender and route of excretion. Following single i.v. doses of ^{14}C-FC-143 (carbonyl carbon labeled) in rats, Johnson et al, found that females excreted virtually all the administered ^{14}C within 1 day. Urinary excretion for males was about 50% of the dose by day 6 and 83% by day 36. Fecal ^{14}C excretion for females was 1.5% by 3 days and for males was 5.4% by 36 days. (Johnson, et al.,

1980) Rapid urinary excretion of ^{14}C following oral doses of ^{14}C-FC-143 was also shown to occur in pregnant rats. (Johnson, et al., 1983)

Excretion rates vary by species studied. Excretion of radiolabeled PFOA was studied in four species by Dupont in 1988. Excretion as a percentage of administered dose 120 hours after dosing was in the following order; female rat, male and female rabbit and male hamster (> 99%); female hamster (60%); male rat (39%); male and female mice (21%). (Dupont, 1988) Of note, the administered dose and routes of exposure and excretion were not specified in this study report.

Rats and dogs respond to pharmacologic interventions that change PFOA excretion rate. In male rats administered single i.v. doses of ^{14}C-FC-143 (carbonyl carbon labeled) cholestyramine (4% w/w in feed) increased cumulative 15 day fecal ^{14}C excretion 9.8-fold versus controls. Total ^{14}C excretion (feces plus urine) was also enhanced, although less dramatically (84.3% of dose vs. 71.8% for controls). (Johnson, et al., 1980a; Johnson JD, et al., 1984) There is no difference between the renal clearances of ^{14}C in male and female dogs either before or after probenecid. Probenecid significantly reduces PFOA clearance in dogs. Glomerular filtration rates of PFOA were similar in rats and dogs. (Hanhijarvi, et al., 1988)

Elimination half-life has been calculated under numerous conditions in rats and dogs. The results show the same differential elimination rate in rats by gender (i.e., female > male), as well as half-life differences by route of exposure and species. Following a single oral dose of ^{14}C-FC-143 (carbonyl carbon labeled) in male rats, the plasma half-life was 4.8 days. (Johnson, et al., 1979)

In female rats administered oral or i.v. doses of ^{14}C-FC-143 (carbonyl carbon labeled), over 90% of the administered dose was recovered in the urine within the first 12 hours. The whole body elimination half-life of PFOA in male and female rats was 15 days and less than one day, respectively, following a single 4-mg/kg intraperineal dose. (Vanden Heuval, et al., 1991) (Johnson, et al., 1983; Johnson, et al., 1980) The half-life of PFOA in the liver was 60 hours for females and 210 hours for male rats. (Ylinen, et al., 1990) The decreased excretion rate (i.e., increased elimination half-life) in males is seen across at least two species: rats (as above) and dogs. The plasma half-life of PFOA was longer in male dogs (473 to 541 hours) than in females (202 to 305 hours). (Hanhijarvi, et al., 1988)

The elimination half-life appears to be similar in male rats exposed to either inhalational or dermal exposure. Following repeated inhalation exposures to FC-143 over a two-week period, blood organic fluoride levels in male rats showed a half-life of five to seven days. (Kennedy, et al., 1986) A blood half-life of five to seven days was seen following repeated dermal exposures in male rats. (Kennedy, 1985)

A calculation of the elimination half-life of an organic fluorine species has been reported in the human. The half-life of total serum organic fluorine in a fluorochemical worker who was removed from further exposure was greater than 18 months. This worker had a blood organic fluorine level of 40 parts per million over the period of one year. The worker's organic fluorine level rose to 70 parts per million for no apparent reason. The worker was then removed from the fluorochemical production area and his blood and urine samples were periodically checked for organic fluorine and PFOA over an 18-month period. A total of 18 months transpired before the

worker's blood organic fluorine level had returned to its base line of 40 parts per million. (Ubel, et al. 1980) Other estimates of serum fluorochemical half-lives have been calculated in the one thousand day range. Ψ This anecdotal account does not actually track the serum PFOA level, but rather the serum total organic fluorine (TOF) level. It was found through empirical means that 90% of the blood organic fluorine existed as PFOA. (Ubel, et al. 1980)

PERFLUOROOCTANE SULFONIC ACID (PFOS or FC-95)

Perfluorooctane sulfonic acid appears to persist in biological systems, being excreted more slowly than PFOA. Single i.v. doses (mean 4.2 mg/kg) of ^{14}C-FC-95 and 0.9% NACL were administered to male rats. By 89 days after dosing, 30.2% of the administered ^{14}C had been excreted in the urine and 12.6% had been excreted in the feces. (Johnson, et al., 1979)

Pharmacologic interventions do appear to increase the excretory rate of PFOS. Fecal and total excretion of ^{14}C were markedly increased in male rats administered Cholestyramine (about 2.7 gm/kg per day) and their diet following single i.v. doses of ^{14}C-FC-95. The results suggest that there was significant enterohepatic circulation of FC-95. (Johnson, et al., 1980a; Johnson, et al., 1984)

Much less is known concerning elimination half-life of PFOS than PFOA; only one calculation of the elimination half-life of PFOS has been attempted. The plasma elimination half-life of ^{14}C following single oral administration of ^{14}C-FC-95 (mean dose 4.2 mg/kg) to male rats was 7.5 days. (Johnson, et al. 1979a)

Ψ Mandel, J. Personal communication.

RESEARCH METHODS

The overall research design is prospective in nature, obtaining multiple serial blood samples from retirees throughout the course of a five-year period. We will track the decline in several serum fluorochemical levels in humans. The health outcomes that will be documented in this study are the half-life determinations of serum ammonium perfluorooctanoate (PFOA), perfluorooctane sulfonic acid (PFOS), perfluorohexane sulfonic acid (PFHS), and serum total organic fluorine (TOF).

High-performance liquid chromatography mass spectrometry/mass spectrometry will be utilized to analyze all serum samples. The accuracy and reliability of this device will be the state of the art at the time of analysis. Periodically, split samples from one or two subjects will be utilized to assess reliability of the analysis.

Only individuals who have retired from 3M Decatur chemical plant from the 1st of January 1995 through the 1st of January 1998, will be included in the study. Using information received from the Human Resources Department of 3M Decatur, 34 individuals were identified to have retired during this time period. Of the 34 individuals identified, 7 have previously been enrolled in the fluorochemical medical surveillance program. Four of these individuals participated in the medical surveillance program in 1994 but not 1997; 3 individuals participated in 1997 only. We will attempt to enroll all 34 retirees into the study. Future retirees will not be enrolled. Serum fluorochemical levels will be drawn every six months. All serum fluorochemical levels will be drawn within a one-month time frame (i.e., within one month of March and September).

Retirees will be recruited to the study through first, an introductory letter and second, a phone conversation from the principle investigator or his designee. Subsequent to the follow-up telephone conversation and if the retiree is willing to enter the study, a written informed consent form (Appendix A) and a medical questionnaire (Appendix B) will be sent to the retiree for his review. Once the retiree signs the consent form, completes the questionnaire, and returns the documents to the principle investigator, he will be entered into the study and scheduled for blood collection. Participation in the study is voluntary and retirees may drop out at any time.

Blood collection will be scheduled biannually, in April and October (to not conflict with Holiday and summer vacation travel). Letters announcing blood collection dates and times will be sent out to each study subject one month prior to each blood collection month. Retirees will update their medical questionnaire at each blood collection. All blood collections will be performed under the supervision of the 3M Decatur plant nurse, Cathy Simpson, RN. Two red-topped tubes will be obtained from each retiree; the 3M Occupational Medicine Service methodology for handling and shipping of fluorochemical blood samples will be applied to all blood samples. Samples will be analyzed by the 3M Environmental Laboratory or its designated contract laboratory. Retirees will receive written reports of their serum fluorochemical levels following each blood collection cycle.

Two methods of calculating the serum fluorochemical half life will be utilized. In the first method, a one compartment model will be assumed and the formula

$$q_t = q_0 (0.5)^{t/t½}$$

will be utilized to estimate the half life of elimination for PFOS, PFOA, PFHS, and TOF. The terms q_t *and* q_0 refer to the serum concentrations at time t and time 0, t is the elapsed time; $t_{1/2}$ is what we seek to solve.

The second method will be used to verify the first. It also assumes a one compartment model. Each retiree's fluorochemical levels will be graphed on a log-linear scale as serum fluorochemical vs. elapsed time from t_0 (in months) and entered into a database. Statistical analysis will be performed to determine the best-fit model that describes the slope of the log-linear line. (Appendix C)

The slope of the log-linear line is related to the elimination constant ($-k_{el}$) through the equation

$$Slope = -k_{el}(2.303)$$

Once the elimination constant is calculated, the half life can be calculated using the relationship

$$t_{1/2} = 0.693/k_{el}$$

Elimination half lives for TOF, PFOS, PFHS, and PFOA will be determined. (Benez, et al. 1985, Medinsky, et al. 1996.)

DISCUSSION

There are a number of limitations associated with the study, including:

1. The lack of pre-study serum fluorochemical levels in the majority of retirees, making it impossible to develop baseline levels for the majority of the study group. The *t0* values will therefore need to be their first fluorochemical level *post-retirement*.

2. Previous studies have performed serum total organic fluorine levels and not serum testing specific for serum PFOS or PFOA; comparability with previous work is thereby compromised.
3. Retirees on medications such as colestipol or cholestyramine, which can artificially increase the rate of elimination of fluorochemicals from the blood will artificially increase the elimination in unpredictable ways, thereby causing an underestimation of the elimination half life in the entire study population.
4. Retirees may unknowingly have medical conditions that will decrease their toxicant elimination rate unpredictably (e.g., renal failure, congestive heart failure, cholelithiasis, etc.), thereby causing an inaccurate estimation of the elimination half life in the entire study population.

For these reasons, the study will occur over the protracted period of five years instead of a shorter time period (i.e., three years). As the only human data plotting total organic fluorine suggest a plasma elimination half-life of 18 months, a three year study would allow for verification of that estimate since three years would be equal to two half-lives. A five-year study, therefore, should include over three half-lives of elimination, and make it possible to use several years' data to calculate the elimination half life.

The overall results collected in this study may be used in publications or in public presentations. Retiree names or other individual data will not be revealed in any publication or other documentations intended for public examination. Individual results will be communicated only to the retiree. Individual results will be considered confidential information and will not be disclosed to anyone outside of the 3M Medical Department without the retiree's written consent.

Quality assurance will be performed per the guidelines set forth in the standard operating procedure for the creation, review, and approval of 3M epidemiology study protocols.

Archiving of study materials, upon completion of the study, will be performed per the standard operating procedure for the creation, auditing, review and approval of 3M epidemiology final reports.

The study will be communicated to the retirees, management, and the technical community. Communications to the retirees will be in the form of a letter, which states the retiree's various blood fluorochemical levels. These letters will be sent out following each blood draw. Communication to management will be in the form of a written document which will undergo the review process as set forth in the standard operating procedure for the creation, auditing, review and approval of 3M epidemiology final reports. The communication to the technical community will be in the form of a scientific paper, which will be submitted to a relevant peer reviewed scientific publication.

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Appendix A

CONSENT FORM FOR COLLECTION OF BLOOD FOR SERUM FLUOROCHEMICAL LEVEL HALF-LIFE DETERMINATION

INTRODUCTION

You and your colleagues were involved in fluorochemical production at 3M Decatur prior to your retirement. You are invited to participate in a research study being conducted by 3M Occupational Medicine Services regarding fluorochemicals. Your participation would involve donating one red-topped test tube of blood approximately every six months for the next five years. Your blood will only be tested for the amount and kind of fluorochemicals it contains; it will not be used to test for any other substances or disease. Please review this consent form carefully and be sure your questions are answered before you make a decision to participate. The plant nurse will be available to provide you with additional information at the time of your medical surveillance blood donation.

PURPOSE OF STUDY

The purpose of the study is to determine how much and what types of fluorochemicals are eliminated from the human body over time.

STUDY PROCEDURES

Your blood will be drawn with one needle stick and require two test tubes. This will occur about every six months for the next five years. A letter will be sent to you in advance informing you of the proper date, time, and place to report to for blood collection.

POTENTIAL RISKS/BENEFITS

The only discomfort you may feel is from the needle stick. You may also have some temporary redness/bruising/swelling in this area after blood collection.

BENEFITS

There will be no direct benefit from your participation in this study. However, the information gained from this study will further help us understand human exposures to fluorochemicals. Your individual results as well as the overall results will be communicated to you only. Your individual results will be considered confidential information and will not be disclosed to anyone outside the 3M Medical Department without your written consent.

COMPENSATION

If you suffer injury or a medical condition that appears to be the result of participating in this study, you will be referred to another health care professional at no cost to you. In the event of a research related injury, compensation will be determined on a case by case basis by 3M.

CONFIDENTIALITY

The overall results collected in this study may be used in publications or public presentation. Your name will not be revealed in any publication or other documents intended for publication examination. Your individual results will be communicated to you only. Your individual results will be considered confidential information and will not be disclosed to anyone outside the 3M Medical Department without your written consent.

SUBJECT RIGHTS/AVAILABILITY OF INFORMATION

If you have any questions about the study now, or later, or in the event of a research related injury or emergency, contact Dr. Jeffrey Mandel (651-733-8670) or Jean Burris, R.N. (651-737-7867). For answers to questions about your rights in regard to this research, you may contact Dr. Larry Zobel, Chair, 3M Institutional Review Board at 651-733-5181.

VOLUNTARY PARTICIPATION AND WITHDRAWAL

Participation in this study is voluntary. Refusal to participate will involve no penalty of loss of benefits to which you are otherwise entitled. You are free to withdraw from the study at any time for any reason. The investigator may stop your participation in this study should it be determined that continued participation may be detrimental to your health. Following each semi-annual blood collection, you will receive a check for $50.00 as compensation for your time and effort.

SUBJECT CONSENT

By signing the consent form, I certify that I am at least 18 years old. I confirm that I have read this consent form, and that I have been given adequate opportunity to ask any questions I may have about this consent form or the study. I also confirm that I understand the scope of my participation in this study, and that all of my questions have been answered to my satisfaction. I am signing this consent form voluntarily, and I desire to participate in the study. I understand that I will receive a copy of this signed consent form. I understand that I will receive a check for $50.00 immediately following each semi-annual collection of my blood.

_____ _____

Signature Date

_____

Printed Name

_____

Witness

Appendix B

MEDICAL HISTORY QUESTIONNAIRE

Please list the names of the **ALL** the medications you take at least once a day.

Please circle the conditions you now suffer from or have suffered from in the last three years.

Kidney Failure

Gall Bladder Disease

Hepatitis/Jaundice

Congestive Heart Failure

Liver Disease

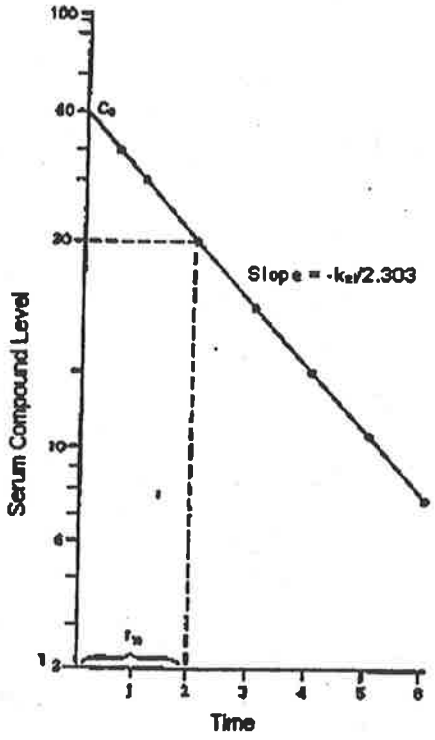
Inflammatory Bowel Disease

Crohn's Disease

Pernicious Anemia

Appendix C

Example of a log-linear plot that will be used to estimate the serum fluorochemical elimination half life in Decatur retirees.



DOCUMENT 48

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Toxicity of Ammonium Perfluorooctanoate in Male Cynomolgus Monkeys after Oral Dosing for 6 Months

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Ammonium perfluorooctanoate (APFO) is a processing aid in the production of fluoropolymers that has been shown to have a long half-life in human blood. To understand the potential toxicological response of primates, groups of male cynomolgus monkeys were given daily po (capsule) doses of either 0, 3, 10, or 30 (reduced to 20) mg/kg/day for 26 weeks. Two monkeys from each of the control and 10 mg/kg/day dose groups were observed for 90 days after the last dose. Clinical observations, clinical chemistry, determination of key hormones, gross and microscopic pathology, cell proliferation, peroxisomal proliferation, bile-acid determination, and serum and liver perfluorooctanoate (PFOA) concentrations were monitored. Toxicity, including weight loss and reduced food consumption, was noted early in the study at the 30 mg/kg/day dose; therefore, the dose was reduced to 20 mg/kg/day. The same signs of toxicity developed in 3 monkeys at 20 mg/kg/day, after which treatment of these monkeys was discontinued. One 30/20 mg/kg/day monkey developed the signs of toxicity noted above and a possible dosing injury, and this monkey was sacrificed *in extremis* on Day 29. A 3 mg/kg/day dose-group monkey was sacrificed *in extremis* on Day 137 for reasons not clearly related to APFO treatment. Dose-dependent increases in liver weight as a result of mitochondrial proliferation occurred in all APFO-treated groups. Histopathologic evidence of liver injury was not observed at either 3 or 10 mg/kg/day. Evidence of liver damage was seen in the monkey sacrificed in moribund condition at the highest dose. Body weights were decreased at 30/20 mg/kg. PFOA concentrations in serum and liver were highly variable, were not linearly proportional to dose, and cleared to background levels within 90 days after the last dose. A no observable effect level was not established in this study, and the low dose of 3 mg/kg/day was considered the lowest observable effect level based on increased liver weight and uncertainty as to the etiology leading to the moribund sacrifice of one low-dose monkey on Day 137. Other than those noted above, there were no APFO-related macroscopic or microscopic changes, changes in clinical chemistry, hormones, or urinalysis, or hematological effects. In particular, effects that have been associated with the development of pancreatic and testicular toxicity in rats were not observed in this study.

Key Words: ammonium perfluorooctanoate; repeated-dose toxicity; cynomolgus monkey; health effects; hepatotoxicity; APFO.

Ammonium perfluorooctanoate (APFO; $C_7F_{15}COO^-NH_4^+$, C.A.S. Registry number 3825-26-1) has been used as a processing aid in the production of fluoropolymers. Perfluorooctanoate (PFOA; $C_7F_{15}COO^-$), the dissociation product of APFO, has been identified in blood samples from exposed workers and the general population (Belisle, 1981; Guy *et al.*, 1976; Hansen *et al.*, 2001; Olsen *et al.*, 1998, 2000; Ubel *et al.*, 1980).

Extensive research into the potential health risk of exposure to APFO was initiated in the late 1970s. Medical monitoring of employees involved in APFO production began in 1976, by measuring serum levels of organic fluorine and performing medical assessments (Ubel *et al.*, 1980). Since the early 1990s, serum concentrations of PFOA have been measured (Olsen *et al.*, 1998, 2000). Eighty percent of the workers had serum PFOA levels less than 6 ppm. Average serum concentrations have been approximately 2 ppm and individual values have ranged from undetectable to 114 ppm.

The potential association of APFO exposure with health effects in fluorochemical production workers has been studied in mortality and clinical studies. No excess cause-specific mortality has been associated with APFO exposure in this workforce (Alexander, unpublished report), and clinical indications of liver and hormonal function, including estradiol, testosterone, and cholecystokinin (CCK), have been normal (Olsen *et al.*, 1998, 2000).

APFO, once dissociated to form PFOA, is not metabolized further (Kuslikis *et al.*, 1992; Vanden Heuvel *et al.*, 1991). There are dramatic sex and species differences in elimination of PFOA (DuPont Haskell Laboratories, 1982; Johnson and Ober, 1980). In male rats, the elimination half-life is approximately 1 week, as opposed to female rats, which clear APFO rapidly with an elimination half-life close to 1 h. Recently developed data from an iv toxicokinetic study in male and female cynomolgus monkeys suggests that the terminal phase elimination half-lives in both sexes of the cynomolgus monkey are approximately 1 month (Butenhoff *et al.*, in preparation). PFOA has been demonstrated to have a long serum elimination half-life in workers (Ubel *et al.*, 1980), which based on recent

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monitoring of retired fluorochemical workers, may be on the order of several years. In addition to urinary excretion, biliary excretion and reabsorption of PFOA are significant, as evidenced by the fact that whole-body elimination was significantly enhanced by cholestyramine administration in APFO-treated male rats (Johnson *et al.*, 1984).

Past dietary studies in rats and mice have demonstrated that the primary target organ for APFO-induced toxicity is the liver (Biegel *et al.*, 2001; Griffith and Long, 1980; Kennedy, 1987; Pastoor *et al.*, 1987). The hepatotoxicity manifests as increased liver weights, hepatocellular hypertrophy, liver degeneration, increases in plasma ALT and AST, necrosis of the liver, proliferation of smooth endoplasmic reticulum and peroxisomes, and hypolipidemia (Haughom and Spydevold, 1992; Intrasuksri *et al.*, 1998; Kawashima *et al.*, 1995; Pastoor *et al.*, 1987; Permadi *et al.*, 1992, 1993). Many of these effects were demonstrated to be reversible when animals were provided with a recovery period. Deaths and weight loss also occurred in these studies, with 100% mortality occurring in both mice and rats at a dietary dose of 3000 ppm.

It is interesting to note that the only previous primate repeat-dose study did not identify the liver as a target organ (Griffith and Long, 1980). In this study, groups of two male and two female rhesus monkeys were treated with APFO by gastric intubation over a 90-day period at dose levels of either 0, 3, 30, or 100 mg/kg. There were no reported effects at 3 mg/kg/day. All monkeys in the 100 mg/kg/day dose group died prior to scheduled sacrifice. One male and both females died at 30 mg/kg prior to scheduled sacrifice, and males and females experienced reductions in body weight at 30 and 100 mg/kg.

APFO is included among several members of a structurally diverse group of compounds that induce peroxisome proliferation and tumors in rat liver, as well as extrahepatic tumors of the pancreas (acinar cells) and/or testis (Leydig cells; Biegel *et al.*, 2001; Cook *et al.*, 1999; Maloney and Waxman, 1999; Reddy and Rao, 1977; Riker Pharmaceuticals, 1987). In a chronic mechanistic dietary study, APFO produced this triad of tumors in rats but was considerably less potent than the model peroxisome proliferator, WY-14,643 (Biegel *et al.*, 2001). Liver tumors produced by peroxisome proliferating compounds may derive from the increased oxidative stress and cell proliferation that accompanies an increase in peroxisomes. However, the Leydig cell and pancreatic acinar cell tumors appear to result from mechanisms other than peroxisome proliferation. Specifically, it is currently thought that Leydig cell tumors in rats associated with peroxisome proliferators may result from the hyperplastic effect of sustained increases in estradiol as a result of induction of aromatase (Biegel *et al.*, 1995, 2001; Cook *et al.*, 1992; Liu *et al.*, 1996a,b). The mechanism for the production of pancreatic acinar cell hyperplasia and tumor formation by certain peroxisome proliferating compounds in rats is less clear. *In vitro* and *in vivo* experiments with WY-14,643 suggest that these exocrine pancreas tumors may be the result of a mild, sustained increase in CCK due to cholestasis (Obourn *et al.*, 1997).

The occurrence of the triad of hepatocellular, pancreatic acinar cell, and Leydig cell tumors associated with peroxisome proliferation is likely to be species specific. The phenomenon of peroxisome proliferation is not uniform across all species. While rats and mice are particularly sensitive to this phenomenon, guinea pigs, cats, dogs, and primates (including humans), are predominantly nonresponsive (Cattley *et al.*, 1999; Kurata *et al.*, 1998; Pugh *et al.*, 2000). A number of compounds that produce Leydig cell tumors in rats fail to do so in mice (Clegg *et al.*, 1997; Cook *et al.*, 1999). There are differences between species in the expression and localization of cholecystokinin receptors that may influence the possible production of pancreatic tumors (Bourassa *et al.*, 1999; Holicky *et al.*, 2001).

The study reported here tested the hypothesis that the male cynomolgus monkey, as a representative primate, does not respond to the effects on the liver, pancreas, and testes that have been associated with APFO and other peroxisome proliferating compounds in the male rat. The specific aims of this study, in addition to observing descriptive toxicity endpoints, were to assess the effect of chronic (26-week) APFO treatment on biological markers associated with the hepatic, pancreatic, and testicular responses seen in the rat with APFO and other peroxisome proliferating compounds. These biological markers included measurement of acyl CoA oxidase activity, replicative DNA synthesis, hormone levels including estradiol and CCK, as well as indications of cholestasis including bilirubin, alkaline phosphatase, and bile acid determination. A three-month recovery phase was added to observe for delayed effects and reversibility of effects. The design of this study, which also included assessment of PFOA concentrations in serum and liver by modern methods of analysis, incorporated significant enhancements over the monkey rhesus study (Griffith and Long, 1980).

MATERIALS AND METHODS

Animals and husbandry. Male cynomolgus monkeys were obtained from Covance Research Products Inc. (Denver, PA) in August 1998. The mean age of the monkeys, as estimated by dentition, was 6 ± 1 (SD) years and ranged from 3 to 9 years. The monkeys weighed 3.2 to 4.5 kg at initiation of treatment. Monkeys were acclimated for 35 days at Covance Laboratories in Madison, Wisconsin before initiation of treatment. Each monkey was assigned a permanent number upon arrival and identified with a collar tag. The monkeys were housed individually in suspended stainless-steel cages. The animal room was environmentally controlled to maintain 18 to 29°C, a relative humidity of 30 to 70%, and a 12-h light/dark cycle.

Certified primate diet (#8726C, Harlan Teklad) was provided once or twice daily along with a variety of fresh and dried fruits as well as various breakfast cereals. Water was provided *ad libitum*. Samples of the water were analyzed for specific microorganisms and contaminants. During the study, several monkeys experiencing low food consumption in the high-dose group were offered supplements to rehydrate the monkeys and stimulate food consumption.

Monkeys were assigned to treatment groups using a computerized blocking

procedure designed to achieve body weight balance within each treatment group.

Materials, dose preparation, and treatment. Ammonium perfluorooctanoate (APFO, lot 332) was provided by 3M (St. Paul, MN; purity 95.2%). Dose levels described here have not been adjusted for purity. Impurities that could contribute to toxicity included perfluorohexanoate (0.73%) and perfluorohepatanoate (3.76%). Gelatin capsules (Torpac, Inc., Fairfield, NJ, Size No. 2, Lot No. 122932) containing the appropriate dose of APFO were used for dose administration. Control monkeys received empty gelatin capsules. Capsules were prepared at least weekly. Individual daily doses were calculated based on the most recently recorded body weights. The dose preparations were stored at room temperature. Since the APFO was added directly to capsules as supplied without the use of a vehicle, no dose analysis was necessary.

Dose levels were 0 (control group, $n = 6$), 3 mg/kg (low-dose group, $n = 4$), 10 mg/kg (mid-dose group, $n = 6$), and 30, later reduced to 20 (30/20) mg/kg on Day 22 (high-dose group, $n = 6$). The capsules were administered orally once a day, 7 days per week, for at least 26 weeks (182 days) except as noted below. One monkey in the low-dose group needed to be replaced for nontreatment-related issues. The replacement monkey (Monkey 5721) received his initial dose on Day 17. High-dose monkeys were initiated on 30 mg/kg; however, due to toxicity, dosing was discontinued from day 12 through 21 and recommenced on Day 22 at 20 mg/kg. Also due to toxicity, dose administration was discontinued for three high-dose monkeys on Day 43 (Monkey 5711), Day 66 (Monkey 5722), and Day 81 (Monkey 5703). Two monkeys in each of the control and mid-dose groups were designated as recovery group monkeys and were observed for reversibility, persistence, or delayed occurrence of toxic effects for 13 weeks after the 26-week treatment period.

Clinical observations. The monkeys were observed twice daily for general health, behavior, and qualitative food consumption. Ophthalmic examinations were conducted on each monkey before treatment and at week 27. Recovery monkeys also were given ophthalmic exams at week 40. Body weights were recorded weekly before initiation of treatment, on the day before initiation of treatment for dose calculation, on the first day of treatment, and weekly thereafter.

Clinical pathology. A clinical laboratory evaluation was conducted before initiation of treatment, on Days 31, 63, 91, and 182 of treatment and during recovery on Days 217, 245, and 275. At each sampling time, blood and urine samples were collected from monkeys that had been fasted overnight. Urine was collected overnight on wet ice before blood sampling; water was provided *ad libitum*. Blood was collected from the femoral vein. Sodium citrate was used as the anticoagulant for coagulation tests, and potassium EDTA was the anticoagulant used for hematology tests. Blood was collected from monkeys with unscheduled sacrifices. Otherwise, monkeys with scheduled collections were bled in random order.

Hematological parameters (blood cell counts [erythrocytes, leukocytes, and platelets], hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration) were measured or calculated using an Abbott Cell-Dyn 3500 hematology analyzer, as were differential cell counts of segmented neutrophils, lymphocytes, eosinophils, and basophils. Blood cell morphology and reticulocyte counts were obtained using a Miller Disk microscopy technique. Bone marrow smears were prepared at euthanization. Coagulation parameters measured were prothrombin time, activated partial thromboplastin time (nephelometry), and fibrinogen.

Clinical chemical analytes were measured on a Hitachi® Model 704,911 Chemistry Analyzer using the Roche Diagnostics, Hitachi® 704,911 Operator Reference Manual, Method Application. These clinical chemistry measurements included glucose (enzymatic/glucose-hexokinase), urea nitrogen (kinetic enzymatic urease/GLDH), creatinine (kinetic—modified Jaffe), total protein (biuret), albumin (bromcresol green), globulin (calculated—total protein minus albumin), total bilirubin (photometric determination of azobilirubin complex after coupling with diazotized sulfanilic acid), cholesterol (kinetic enzymatic without extraction using Trinder reaction), triglycerides (kinetic enzymatic without blank), aspartate aminotransferase (kinetic—NADH consumption with oxaloacetate), alanine aminotransferase (kinetic—NADH consumption with pyruvate), alkaline phosphatase (kinetic using p-nitrophenyl-phosphate), gamma-glutamyl transferase (kinetic using glutamyl-p-nitroanilide), sorbitol dehydrogenase (D-fructose NADH UV), creatine kinase (kinetic using N-acetyl cysteine), calcium (cresolphthalein complexing), inorganic phosphorus (UV—phosphomolybdate), sodium (ion-selective electrode—indirect), potassium (ion-selective electrode—indirect), chloride (ion-selective electrode—indirect), total bile acids (enzymatic), amylase (enzymatic), lipase (enzymatic), and pancreatic-specific amylase (enzymatic).

Urine was analyzed for color, clarity, volume (approximately 16 h overnight), specific gravity, pH, protein, glucose, ketones, bilirubin, blood, urobilinogen, microscopic examination of sediment, and general appearance. Color and clarity were determined by observation. Volume was determined to the nearest ml in a graduated cylinder. Specific gravity was determined on an AO/TS refractometer (temperature compensated) using one large drop of urine. Measurement of pH, protein, glucose, ketones, bilirubin, blood, and urobilinogen was accomplished colorimetrically with Multistix® strips. After color, clarity, volume, specific gravity, and Multistix determinations, the urine was centrifuged, the supernatant poured off, and a drop of sediment placed on a glass slide. The slide was scanned under low power light microscopy for crystals and casts and then under high power to count other sediment constituents.

Determination of hormones. Blood was collected from the femoral vein of nonfasted monkeys 18, 8, and 4 days prior to initiation of treatment (Study Days −18, −8, and −4) and on Days 35, 66, 94, and 183 of treatment. Blood collection from recovery monkeys occurred on Study Days 220, 248, and 276 after initiation of treatment. Blood samples were split into serum and plasma samples for further analysis for various hormones. Plasma samples were analyzed for cholecystokinin and testosterone (with the exception of recovery monkeys in which case serum samples were used for testosterone). Serum was analyzed for estradiol, estrone, estriol, thyroid stimulating hormone, total and free triiodothyronine, and total and free thyroxine.

Serum levels of cortisol, testosterone, estradiol, estrone, estriol, free and total triiodothyronine (FT_3 and TT_3) and free and total thyroxine (FT_4 and TT_4) were measured at Anilytics, Inc., Gaithersburg, MD, using radioimmunoassay methods under Anilytics, Inc. standard operating procedures. ICN Pharmaceuticals radioimmunoassay (RIA) kits were used for determinination of cortisol, testosterone, estradiol, and estrone. Total estriol was measured using a Coat-A-Count Total Estriol kit (Diagnostic Products Corporation, Los Angeles, CA). Thyroid stimulating hormone (TSH) was analyzed by an in-house method at Anilytics, Inc. (Gaithersburg, MD) employing a double-antibody RIA procedure using human reagents (Anilytics, Inc. Standard Operating Procedure for Non-Human Primate Thyroid Stimulating Hormone [TSH], May 12, 1998). FT_3, TT_3, FT_4, and TT_4 were determined using Coat-A-Count RIA kits (Diagnostic Products Corporation, Los Angeles, CA).

Frozen plasma samples were sent to Haskell Laboratory for CCK analysis. Plasma samples were extracted over a C-18 Sep Pak column (Supelco Co., Bellefonte, PA) washed with 0.05% trifluoroacetic acid (TFA), and eluted with 50% acetonitrile/50% TFA. Extracted samples were dried under vacuum and reconstituted prior to analysis by RIA (Obourn *et al.*, 1997).

Liver biochemistry. Samples of the right lateral lobe of the livers from each monkey, scheduled and unscheduled sacrifices, were flash-frozen in liquid nitrogen at necropsy and analyzed for palmitoyl CoA oxidase activity by the method of Bronfman *et al.* (1979).

Bile (up to 5 ml) was collected at sacrifice of each monkey, flash frozen in liquid nitrogen and analyzed for specific bile acids. Bile samples were stored frozen (−70°C) prior to analysis. After thawing, bile acids were extracted from a 20 μl aliquot of bile using solid phase extraction on Isoelute C18 cartridges. The extracted samples were reconstituted in HPLC grade methanol, filtered through 0.45μ nylon filter, and subjected to HPLC analysis on a C18 reversed phase column (25 cm, Apex ODS 5 m) using a 75% (v/v) methanol/ 25% (v/v) potassium phosphate buffer (10 mM, pH 5.44) mobile phase. Bile acids

(taurocholic acid, glycocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, taurolithocholic acid, and glycolithocholic acid) were quantified against authentic standards by UV detection at 200 nm using Chromeleon software.

Determination of serum and liver PFOA content. Approximately 2 ml of whole blood (for serum) were collected from the femoral vein of nonfasted monkeys during Study Week 2 and every two weeks thereafter.

A section of liver was collected at sacrifice from each monkey, weighed, flash frozen in liquid nitrogen, and stored at −60 to −80°C until shipped on dry ice to 3M for analysis.

PFOA levels in sera and liver were determined using an ion-pairing extraction followed by quantitative tandem mass spectrometry; the method is described in detail elsewhere (Hansen *et al.*, 2001). Briefly, the serum or liver sample was thoroughly mixed with a buffered solution of tetra-butyl ammonium hydrogen sulfate. The PFOA ion pair was partitioned into methyl *tert*-butyl ether, an exact volume of which was removed from the solution, concentrated under nitrogen, and reconstituted in methanol. Liver samples were homogenized in water prior to extraction.

The extracts were analyzed by high performance liquid chromatography electrospray tandem mass spectrometry in the negative ion multiple response monitoring mode. A characteristic product ion produced by fragmentation of the primary ion was monitored quantitatively and evaluated versus an extracted curve weighted 1/x.

Concurrent with blood collections for PFOA determination, at least 2 ml of urine was collected on wet ice and at least 5 g of feces (overnight) were collected from each monkey. Results of urine and fecal analysis will be discussed in a future paper.

Anatomic pathology. After 26 weeks of treatment, 4 monkeys in the control group, the three surviving monkeys in the 3 mg/kg dose group, 4 monkeys in the 10 mg/kg dose group, and the 5 surviving monkeys in the 30/20 mg/kg dose group were fasted overnight, anesthetized with ketamine and xylazine, weighed, exsanguinated, and necropsied. Similarly, the two monkeys in the control and 10 mg/kg dose recovery groups were sacrificed 13 weeks following the last dose on Day 182. Necropsies were also performed on the 3 mg/kg and 30/20 mg/kg dose-group monkeys that had unscheduled sacrifices on treatment Days 137 and 79, respectively. The adrenals, brain, epididymis, kidneys, liver, pancreas, testes, and thyroids with parathyroids were collected and weighed. Organ to body weight and organ to brain weight ratios were calculated.

The following were collected for microscopic evaluation: adrenal, aorta, brain, cecum, colon, duodenum, epididymis, esophagus, eyes, femur, gall bladder, heart, ileum, jejunum, kidney, lesions, liver, lung, mammary gland, mesenteric lymph node, pancreas, pituitary, prostate, rectum, salivary gland (mandibular), sciatic nerve, seminal vesicles, skeletal muscle (thigh), skin, spinal cord, spleen, sternum with bone marrow, stomach, testes, thyroid, trachea, and, urinary bladder. Tissues were embedded in paraffin, stained with hematoxylin and eosin, and examined by light microscopy.

Replicative DNA synthesis. Representative samples of the left lateral lobe of the liver, left and right testes and pancreas were collected from each monkey. After fixation (formalin or zinc formalin), samples were embedded in paraffin, and sections (~5 μm) were prepared. Previously described methods were used to stain tissues for PCNA (Eldridge *et al.*, 1993). Briefly, tissue sections were incubated with a monoclonal antibody to PCNA (Lot # 107 DAKO, Carpenteria, CA) and reagents required for the avidin-biotin peroxidase method (MslgG Kit Lot # PK6102 Vector, Burlingame, CA) for the detection of the antigen-antibody complex. PCNA expression in cells in all stages of the cell cycle was localized by the chromagen, 3,3′-diaminobenzidine (DAB Lot # 108H8210, Sigma Chemical Co., St. Louis, MO). Tissue sections were counterstained with hematoxylin. Liver cell proliferation was determined by scoring the percent of cells in S-phase among at least 3000 cells in ten randomly selected fields per monkey. For the pancreas, islet and exocrine cells on an entire tissue section were observed and scored subjectively with "3" representing cases in which exocrine cells stained greater than islets and "4" representing cases in which islets and exocrine cells were heavily stained. In the case of the testes, the tissue section furthest from the slide label was scored and the percentage of Leydig cells in S-phase was determined by scoring at least 100 cells on each section.

Statistics. Differences from control values were determined by one-way ANOVA, if applicable, and where significant, followed by Dunnett's test used for control versus treated group comparisons. Paired *t*-tests were used to compare each treatment mean to its baseline value. One-way analysis of covariance (ANCOVA) was used to analyze body weights with initial body weight as the covariate. *p*-values less than 0.05 were considered to be statistically significant. The highest biological significance was attributed when both the within-dose group and time-related pair-wise comparisons were both statistically significant. For interpretive purposes, palmitoyl CoA oxidase activities less than 2-fold greater than control activities were not considered to represent an adverse effect and were considered to reflect natural variation. Only data collected on or after the first day of treatment were analyzed statistically. Data collected before the first day of treatment or during recovery (except for hormone analyses data) were not analyzed statistically.

RESULTS

Animals and Husbandry

One 3 mg/kg/day dose-group monkey was sacrificed on Day 14 due to bacterial septicemia, a condition not believed to be related to treatment. This monkey was replaced on Day 15. The replacement monkey began dosing on Day 17.

Clinical Observations

Overall, body-weight changes as a percent of initial prestudy body weight in the 30/20 mg/kg dose group were significantly lower than controls over the 27 week period (Fig. 1). During the first week of dosing, all monkeys in the 30 mg/kg/day dose group had qualitatively low food consumption and lost from 3.1 to 7.5% of body weight. Four of the 6 monkeys also had few or no feces. Dosing was suspended on Day 12. On Day 22, dosing was reinitiated at a lower dose, 20 mg/kg/day. After commencing the 20 mg/kg/day dosing on Day 22, weight change as a percent of prestudy weight continued to be significantly depressed compared to the other groups (Fig. 1). Two monkeys in this group remained on this regimen for the intended 6-month dosing period. By the end of the dosing period, these two monkeys showed a mean gain in body weight of 5% (individually, −8 and 18%) over their predosing weight, compared to 19, 20, and 20% for the control, 3 mg/kg, and 10 mg/kg groups, respectively (Fig. 1). Dosing of three monkeys in this group was discontinued on Days 43, 66, or 81, respectively. These three monkeys exhibited low or no food consumption, few or no feces, and dramatic body-weight losses (18–23%) prior to being suspended permanently from dosing (Fig. 2). These three monkeys regained their lost weight over the remainder of the study and experienced gains over their predosing weights of 5, 16, and 29% (Fig. 2). The sixth monkey in this group was sacrificed in moribund condition on Day 29. In addition to the signs noted above for the other monkeys in this group, this monkey had lost 12.5% of body weight, was notably hypoactive, and cold to the touch.

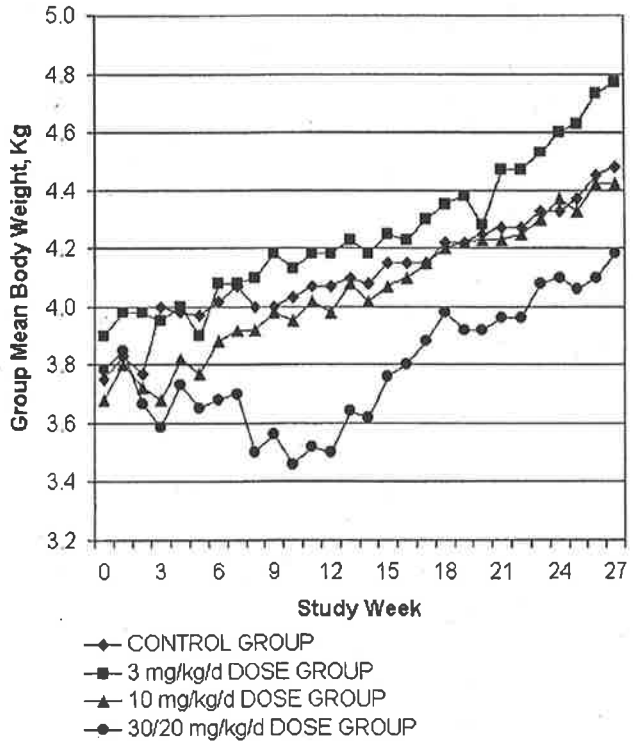

FIG. 1. Group mean body weight for male cynomolgus monkeys through 26 weeks of daily po capsule dosing with ammonium perfluorooctanoate. Monkeys in the 30/20 mg/kg/day dose group experienced toxicity, including weight loss, in the first weeks of the study after dosing with 30 mg/kg/day, and this toxicity continued for 4 of the 6 monkeys in this dose group after reducing the dose to 20 mg/kg/day (see Fig. 2).

Clinical signs, food consumption, and body weights were normal in all monkeys receiving 3 and 10 mg/kg with the exception of one anomalous occurrence in a 3 mg/kg monkey. This low-dose monkey was sacrificed in moribund condition on Day 137. In addition to few feces, low food consumption, and loss of approximately 10% body weight in 1 week, this monkey exhibited hind-limb paralysis, ataxia, and lack of response to a painful stimulus in the week prior to sacrifice. No ophthalmic effects in any of the APFO-treated monkeys were noted.

Two control and two 10 mg/kg dose-group monkeys were observed for delayed effects for 13 weeks after the end of the 26-week dosing period. The 10 mg/kg dose-group recovery monkeys experienced body-weight changes of 5 and −3% of Week 27 (end of dosing period) weights as compared to the control recovery monkeys, who experienced weight gains of 10 and 11% of their Week 27 weights (Fig. 3).

Clinical Pathology

With the exception of individual observations and small but significant increases in triglycerides noted on Days 31, 63, and 91 in the 30/20 mg/kg/day dose group, no major findings were present in the clinical chemistry, hematology, and urinalysis that could be attributed to treatment. The group mean data for glucose, cholesterol, triglycerides, alkaline phosphatase, alanine transaminase, and total bilirubin are presented in Table 1.

Administration of APFO at 3 or 10 mg/kg produced no apparent effects on measured clinical parameters including hematology, coagulation, clinical chemistry, and urinalysis. However, the 3 mg/kg/day dose group monkey that was sacrificed in moribund condition on Day 137 showed marked hyperfibrinogenemia, moderate lymphopenia, moderate hypoalbuminemia, and mild hypocholesterolemia. It should be noted that 11 days prior to initiation of treatment (Day −11), this monkey had a high hematocrit (49.7%) and albumin concentration (5.6 g/dl), possibly indicative of mild dehydration, and it had the lowest neutrophil count (1600/μl) of all monkeys.

Findings in the 30/20 mg/kg/day dose group were compli-

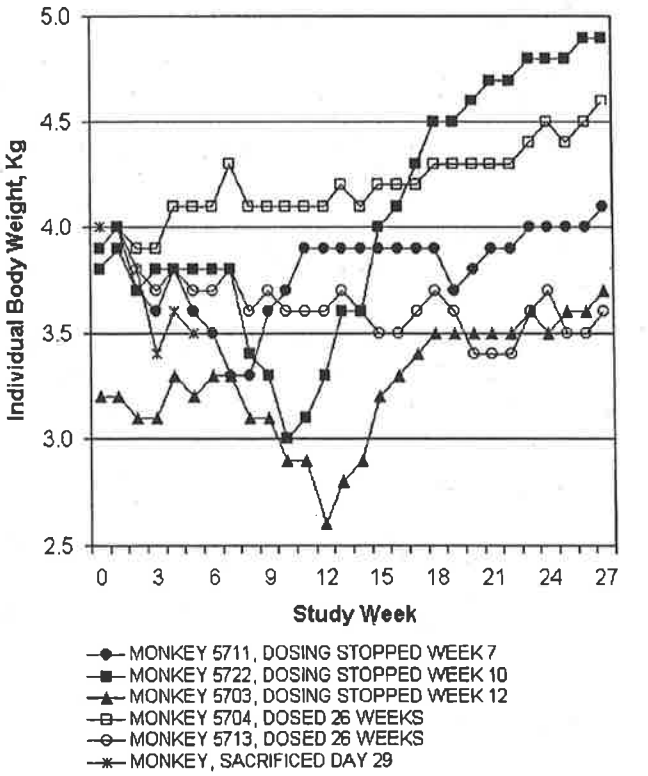

FIG. 2. Individual body weight for male cynomolgus monkeys during daily po capsule dosing with 30/20 mg/kg/day ammonium perfluorooctanoate. Monkeys in the 30/20 mg/kg/day dose group experienced toxicity, including weight loss, in the first weeks of the study after dosing with 30 mg/kg/day. Dosing was suspended during study week 2 and started again at 20 mg/kg/day at the beginning of study week 4. One monkey in this group was sacrificed in moribund condition at the beginning of week 5, and dosing was stopped due to excessive toxicity, including weight loss, for three other monkeys in the 30/20 mg/kg/day dose group in weeks 7, 10, and 12, respectively. The latter three monkeys regained body weight after dosing was stopped. Only two monkeys in the 30/20 mg/kg/day dose group were treated through study week 26.

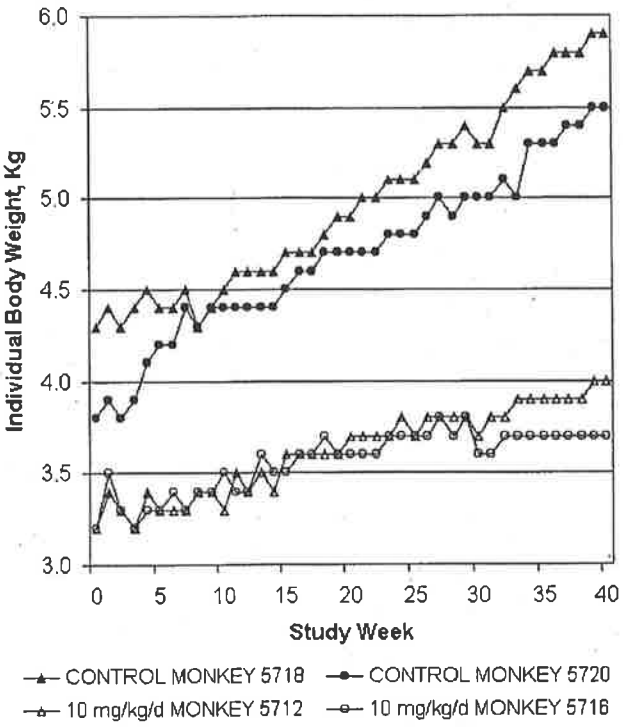

FIG. 3. Individual body weight for control and 10 mg/kg/day dose group male cynomolgus monkeys during 26 weeks of po capsule dosing and 14 weeks of posttreatment recovery.

cated by the unscheduled sacrifice of one monkey on Day 29 and the cessation of treatment for three others by Day 81. Only two monkeys remained on treatment during clinical pathology testing intervals on Days 91 and 182. In this small group, mild increases in triglycerides and mild to marked decreases in absolute neutrophil count, total protein concentration, and albumin concentration occurred. The differences in triglycerides were statistically significant on Days 31 and 182 versus pretreatment values. The other differences were not statistically significant but were consistent over time. Absolute neutrophil counts and albumin concentrations were mildly decreased prior to cessation of treatment for the three monkeys for which dosing was suspended. On Day 63, the 30/20 mg/kg dose-group monkey for which daily dosing was stopped on Day 66 had approximately 10-fold increases over the means in serum activities of aspartate aminotransferase (419 IU/l), alanine aminotransferase (595 IU/l), and creatine kinase (1122 IU/l), as well as a 5-fold increase in sorbitol dehydrogenase (20 IU/l) and an increase in serum bile acids. The monkey that was sacrificed in moribund condition on Day 29 had markedly elevated serum activities of aspartate aminotransferase (1974 IU/l), alanine aminotransferase (1463 IU/l), sorbitol dehydrogenase (59 IU/l), and creatine kinase (68,850 IU/l) as well as markedly low cholesterol (14 mg/dl).

Hormone Determinations

Data for thyroid hormones, estradiol, testosterone, and cholecystokinin are presented in Table 2. No effects on estrone, estriol, or testosterone were noted. Mean group estradiol values in all groups including the controls tended to be considerably lower during the treatment period than the corresponding pretreatment values. These values appeared to be lower in the high-dose group monkeys than in the other groups. The estradiol values in the recovery monkeys tended to be similar to the predose values. No significant changes in testosterone values were seen during the treatment period. In the last recovery sampling, mean values (9 and 15 ng/ml) tended to be higher than means during the rest of the study (1–8 ng/ml).

TSH and free and total thyroxine (FT_4 and TT_4) were not altered in a significant manner during the study. In all groups, FT_3 and TT_3 values taken on predosing Days −18 and −8 (data not shown) were considerably higher than those obtained on predosing Day −4 and throughout the remainder of the dosing period; therefore, the Day −4 values were considered the most appropriate prestudy values for comparison. With free and total triiodothyronine (FT_3 and TT_3), mean group values from predosing Day −4 to the end of the study remained relatively constant; although, monkeys in the 30/20 mg/kg dose group exhibited generally lower group mean values. The mean individual changes in FT_3 and TT_3 over the period that each monkey was being dosed were decreased compared to controls in a statistically significant manner in the 30/20 mg/kg/day dose group. For the three 30/20 mg/kg/day dose-group monkeys suspended from dosing, FT_3 and TT_3 tended to increase toward prestudy values after suspension of dosing.

No biologically significant alterations in relation to dose groups in plasma CCK concentrations were observed.

Liver Biochemistry

Hepatic DNA content and enzymes that are specific markers for different subcellular fractions were determined in an attempt to explain the increased liver weights observed in these studies. APFO administration resulted in a small increase in peroxisomal CN^--insensitive palmitoyl CoA oxidation and a marked increase in mitochondrial succinate dehydrogenase activity. No biologically significant effects were seen on acid phosphatase or glucose-6-phosphatase activities; these are markers for lysosomes and endoplasmic reticulum respectively (Table 3). Additionally, there appeared to be a dose-related decrease in hepatic DNA concentration. There were no significant differences between control and treated groups with respect to the amount and distribution of bile acids (Table 4).

PFOA Concentration in Serum and Liver

Although considerable variability in measurement occurred, steady-state values for PFOA in the serum appeared to have been attained with 4 to 6 weeks of daily dosing. Serum con-

TABLE 1
Clinical Chemistries in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

	Treatment week (day)				
	−2 (−11)	5 (31)	10 (63)	14 (91)	27 (182)
Glucose (mg/dl)					
Control	93 ± 15	78 ± 28	78 ± 21	79 ± 12	71 ± 14
3 mg/kg/day	88 ± 12[a]	78 ± 9	68 ± 7	78 ± 10	63 ± 8[b]
10 mg/kg/day	92 ± 22	73 ± 9	71 ± 11	77 ± 9	66 ± 20
30/20 mg/kg/day	81 ± 5	75 ± 9[c]	76 ± 7[d]	85 ± 19[e]	81 ± 28[e]
Cholesterol (mg/dl)					
Control	147 ± 44	146 ± 19	151 ± 37	167 ± 45*	156 ± 35
3 mg/kg/day	174 ± 54	151 ± 60	161 ± 61	157 ± 51	142 ± 47
10 mg/kg/day	145 ± 22	142 ± 26	158 ± 46	155 ± 31	154 ± 31
30/20 mg/kg/day	140 ± 26	158 ± 21	146 ± 12	142 ± 9*	150 ± 16
Triglycerides (mg/dl)					
Control	39 ± 11	44 ± 23	43 ± 14	40 ± 9	44 ± 9
3 mg/kg/day	50 ± 10	51 ± 24	59 ± 20	56 ± 25	51 ± 24
10 mg/kg/day	61 ± 18**	76 ± 27*	76 ± 27*	88 ± 37*,**	72 ± 25
30/20 mg/kg/day	57 ± 13	108 ± 57*,**	67 ± 39	99 ± 55	92 ± 40**
Alkaline phosphatase (IU/l)					
Control	455 ± 202	577 ± 232*	582 ± 193*	529 ± 240	444 ± 191
3 mg/kg/day	556 ± 180	629 ± 194	612 ± 170*	585 ± 178	574 ± 290
10 mg/kg/day	604 ± 174	704 ± 165*	668 ± 190	656 ± 209	544 ± 181
30/20 mg/kg/day	521 ± 232	551 ± 285	484 ± 160	432 ± 306	384 ± 261
Alanine transaminase (IU/l)					
Control	58 ± 29	49 ± 17	62 ± 24	63 ± 23	68 ± 16
3 mg/kg/day	42 ± 8	29 ± 6	45 ± 27	47 ± 25	43 ± 6
10 mg/kg/day	77 ± 53	55 ± 23	53 ± 19	53 ± 16	53 ± 27
30/20 mg/kg/day	67 ± 42	36 ± 14	192 ± 270	48 ± 18	49 ± 8
Total bilirubin (mg/dl)					
Control	0.0 ± 0.1	0.2 ± 0.1	0.5 ± 0.2*	0.2 ± 0.2*	0.3 ± 0.1*
3 mg/kg/day	0.1 ± 0.2	0.1 ± 0.1	0.3 ± 0.1*	0.3 ± 0.1*	0.2 ± 0.2
10 mg/kg/day	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1**	0.2 ± 0.1	0.3 ± 0.2
30/20 mg/kg/day	0.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	0.1 ± 0.1	0.3 ± 0.4

Note. Group means ± SD. Statistics reflect only those animals receiving treatment when blood was drawn, including comparisons to prestudy values. Control group and 10 mg/kg/day group, $n = 6$; 3 mg/kg/day group, $n = 4$, unless otherwise noted; 30/20 mg/kg/day group, $n = 6$, unless otherwise noted.

[a] Monkey 5721 was added to study in Week 3 (Day 17) to replace a monkey (5723).

[b] $n = 3$. Monkey 5721 was sacrificed in moribund condition in Week 20 (Day 137).

[c] $n = 5$. Monkey 5724 was sacrificed in moribund condition in Week 5 (Day 29).

[d] $n = 4$. Dosing of monkey 5711 was suspended in Week 7 (Day 43).

[e] $n = 2$. Dosing of monkeys 5722 and 5703 was suspended in Weeks 10 (Day 66) and 12 (Day 81), respectively.

*Significantly different from pretreatment values (week 2) by a two-tailed, paired Student's t-test ($p < 0.05$)

**Significantly different from time-related control using Dunnett's t-test ($p < 0.05$, 2 tailed).

centration of PFOA did not increase in a linear dose-dependent manner. Assuming that measurements made at or after Week 6 represent repeat measures at steady state, mean serum concentrations ± SD (range of values, number of samples) for the 3 mg/kg, 10 mg/kg, and 30/20 mg/kg dose groups were 77 ± 39 μg/ml (10–154, 44), 86 ± 33 μg/ml (10–180, 70), and 158 ± 100 μg/ml (20–467, 33) μg PFOA/ml, respectively. Two-thirds (43/64) of the control monkey serums in this time period had detectable PFOA above the limit of quantitation of 0.014 μg/ml, and these averaged 0.203 ± 0.154 μg/ml. The mean values for the 3 mg/kg and 10 mg/kg dose groups were not significantly different at the $p = 0.05$ level. However, the mean for the 30/20 mg/kg/day dose group was significantly different from both lower dose groups ($p < 0.001$). In the recovery group monkeys (10 mg/kg), serum PFOA concentrations returned to baseline by the end of the 90-day recovery period.

Liver tissue PFOA concentrations measured in the monkeys are represented in Table 5. As with serum, the PFOA concentrations in liver did not increase in linear proportion to dose. The values at terminal sacrifice just after dosing for the 3 mg/kg and 10 mg/kg dose groups were similar, and ranged from 6.29 to 21.9 μg PFOA/g tissue. The 30/20 mg/kg/day dose-group values for the two monkeys sacrificed on Day 183 were 16.0 and 83.3 μg PFOA/g tissue. It is noteworthy that the highest liver tissue value obtained (154 μg PFOA/g tissue) was from the monkey sacrificed *in extremis* on Day 29. Liver tissue

TABLE 2
Hormones in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

	Treatment week (day)				
	−1 (−4)	5 (35)	10 (66)	14 (94)	27 (183)
Thyroid stimulating hormone (mU/ml)					
Control	0.37 ± 0.36	0.23 ± 0.23	0.23 ± 0.34	0.54 ± 0.46	0.40 ± 0.23
3 mg/kg/day	0.53 ± 0.58[a]	0.36 ± 0.53	0.19 ± 0.27	0.60 ± 0.55	0.65 ± 0.17[b]*
10 mg/kg/day	0.46 ± 0.57	0.26 ± 0.44	0.56 ± 1.07	0.38 ± 0.53	0.87 ± 1.09*
30/20 mg/kg/day	0.29 ± 0.28	0.03 ± 0.04[c]	0.22 ± 0.25[d]	0.20 ± 0.11[e]	0.39 ± 0.10[e]
Total thyroxine (μg/dl)					
Control	5.13 ± 0.81	4.72 ± 0.95	4.65 ± 0.86	4.22 ± 0.90	3.84 ± 0.77
3 mg/kg/day	5.16 ± 1.28	4.02 ± 0.76	3.52 ± 0.53	3.55 ± 0.38	2.58 ± 0.17**
10 mg/kg/day	4.17 ± 0.51	2.95 ± 0.46**	3.04 ± 0.46**	2.93 ± 0.42**	2.71 ± 0.35**
30/20 mg/kg/day	4.51 ± 0.79	3.70 ± 0.29	3.27 ± 1.02**	3.76 ± 0.42	2.61 ± 0.25**
Free thyroxine (μg/dl)					
Control	1.77 ± 0.29	1.60 ± 0.34	1.48 ± 0.26	1.49 ± 0.50	1.55 ± 0.43
3 mg/kg/day	1.72 ± 0.35	1.47 ± 0.16	1.34 ± 0.30	1.32 ± 0.28	1.04 ± 0.04
10 mg/kg/day	1.56 ± 0.18	1.09 ± 0.17**	1.08 ± 0.20**	1.06 ± 0.20	0.96 ± 0.13**
30/20 mg/kg/day	1.56 ± 0.26	1.23 ± 0.06**	1.08 ± 0.26	1.34 ± 0.27	0.90 ± 0.24**
Total triiodothyronine (ng/dl)					
Control	155 ± 18	171 ± 16	163 ± 19	162 ± 25	157 ± 15
3 mg/kg/day	150 ± 26	168 ± 30	170 ± 21*	177 ± 28*	134 ± 17
10 mg/kg/day	170 ± 23	152 ± 15	162 ± 10	157 ± 18	135 ± 23
30/20 mg/kg/day	148 ± 16	110 ± 39**	90 ± 51**	120 ± 35	104 ± 33**
Free triiodothyronine (ng/dl)					
Control	6.39 ± 0.63	5.49 ± 0.70	5.71 ± 1.14	5.02 ± 0.73	5.62 ± 0.89
3 mg/kg/day	6.06 ± 0.63	5.58 ± 0.65	6.23 ± 0.59	5.28 ± 0.34	4.87 ± 0.12
10 mg/kg/day	6.16 ± 0.44	5.13 ± 0.41	5.31 ± 0.54	4.98 ± 0.89	4.67 ± 0.64
30/20 mg/kg/day	6.00 ± 0.88	3.78 ± 1.18**	3.01 ± 1.96**	4.46 ± 0.76	3.39 ± 1.54**
Testosterone (ng/ml)					
Control	3.76 ± 3.46	2.22 ± 2.63	4.76 ± 3.45	4.63 ± 4.31	7.49 ± 4.62
3 mg/kg/day	6.67 ± 6.55	3.03 ± 2.99	3.68 ± 2.26	7.36 ± 2.66	7.81 ± 4.27
10 mg/kg/day	2.47 ± 2.42	2.00 ± 2.02	5.15 ± 3.92	2.89 ± 2.06	7.83 ± 3.69*
30/20 mg/kg/day	3.97 ± 3.21	0.81 ± 0.49	2.76 ± 3.01	1.25 ± 0.09	1.74 ± 0.44
Estradiol (pg/ml)					
Control	24.9 ± 6.3	7.6 ± 7.0	15.0 ± 12.5	13.5 ± 10.1	10.8 ± 17.0
3 mg/kg/day	35.1 ± 9.7	14.7 ± 12.7	18.3 ± 11.5	7.7 ± 7.8	13.6 ± 11.1
10 mg/kg/day	30.2 ± 7.4	11.2 ± 7.4	19.8 ± 10.1	6.4 ± 7.9	7.8 ± 6.2
30/20 mg/kg/day	27.7 ± 6.7	4.6 ± 7.1	2.1 ± 4.2	0.0 ± 0.0	0.0 ± 0.0
Cholecystokinin (Fmol/mR)					
Control	1.76 ± 0.55	3.15 ± 0.86*	3.07 ± 1.52*	1.79 ± 0.59	2.43 ± 0.84*
3 mg/kg/day	1.81 ± 0.82	3.02 ± 1.28*	3.90 ± 2.69	1.38 ± 0.73[f]	3.03 ± 1.42
10 mg/kg/day	1.53 ± 0.62	3.10 ± 1.18*	2.74 ± 1.30	1.43 ± 0.23[g]	2.31 ± 0.88
30/20 mg/kg/day	1.88 ± 1.07	2.55 ± 0.39	2.44 ± 1.10	1.90 ± 0.14	1.80 ± 1.16

Note. Group means ± SD. Statistics reflect only those animals receiving treatment when blood was drawn, including comparisons to prestudy values. Control group, $n = 6$; 3 mg/kg/day group, $n = 4$, unless otherwise noted; 10 mg/kg/day and 30/20 mg/kg/day groups, $n = 6$, unless otherwise noted.

[a]Monkey 5721 was added to study in Week 3 (Day 17) to replace a monkey (5723).

[b]$n = 3$. Monkey 5721 was sacrificed in moribund condition in Week 20 (Day 137).

[c]$n = 5$. Monkey 5724 was sacrificed in moribund condition in Week 5 (Day 29).

[d]$n = 4$. Dosing of monkey 5711 was suspended in Week 7 (Day 43).

[e]$n = 2$. Dosing of monkeys 5722 and 5703 was suspended in Weeks 10 (Day 66) and 12 (Day 81), respectively.

[f]$n = 3$. Technical problems were encountered with extraction of sample from Monkey 5706.

[g]$n = 5$. Technical problems were encountered with extraction of sample from Monkey 5710.

*Significantly different from pretreatment values (Week −1) by a two-tailed, paired Student's t-test ($p < 0.05$).

**Significantly different from time-related control using Dunnett's t-test ($p < 0.05$, 2 tailed).

PFOA concentrations from 10 mg/kg dose-group monkeys sacrificed at the end of the recovery period or from the three 30/20 dose-group monkeys that were removed from dosing by Day 81 and sacrificed on Day 183 ranged from 0.08 to 1.41 μg PFOA/g tissue. At the recovery sacrifice of the 10 mg/kg monkeys, liver PFOA concentrations had returned to normal

TABLE 3
Subcellular Marker Enzyme Activities in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

Group, mg/kg (n)	DNA	SDH	PCO	AP	G6P
0 (4)	1.44 ± 0.28	0.21 ± 0.15	0.53 ± 0.12	0.78 ± 0.10	12.32 ± 3.11
3 (3)	1.23 ± 0.89	1.77 ± 1.59	0.47 ± 0.13	0.81 ± 0.11	6.02 ± 0.33*
10 (4)	1.25 ± 0.37	0.55 ± 0.14	0.90 ± 0.29	0.80 ± 0.14	10.17 ± 0.63
30/20 (2)	1.02 ± 0.17*	1.37 ± 0.73*	1.36 ± 0.34*	0.55 ± 0.10	8.83 ± 1.41
Recovery					
0 (2)	1.69 ± 0.13	0.48 ± 0.43	0.39 ± 0.00	0.70 ± 0.06	4.59 ± 1.15
10 (2)	1.16 ± 0.15	1.55 ± 1.83	0.46 ± 0.16	0.76 ± 0.10	7.75 ± 1.63

Note. Group means ± SD. DNA content given in mg DNA/g liver. SDH, Mitochondrial marker; succinate dehydrogenase, μmol cytochrome c reduced/min/g liver. PCO, peroxisomal marker; CN^--insensitive palmitoyl CoA oxidation, μmol/min/g liver. AP, lysosomal marker; acid phosphatase, μmol/min/g liver. G6P, endoplasmic reticulum marker; glucose-6-phosphatase, μmol/min/g liver.

*Significantly different from control (0 mg/kg) group, $p > 0.05$.

(0.08 and 0.15 μg PFOA/g tissue compared to control range of less than the limit of quantitation to 0.23 μg PFOA/g tissue).

PFOA was excreted in urine and feces (data not shown).

Anatomic Pathology

Increases occurred in mean absolute liver weights and mean liver-to-body weight percentages in all dose groups at terminal sacrifice after 26 weeks of dosing (Table 6). Absolute liver weight was significantly elevated ($p < 0.01$) in all treated groups relative to control. Relative liver weight increases showed a positive dose-response trend, and was significantly higher than controls in the two 30/20 mg/kg/day dose-group monkeys who were dosed through scheduled termination ($p <$

TABLE 4
Bile Acids (μmol/ml) in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

	Control ($n = 4$)	3 mg/kg/day ($n = 4$)[a]	10 mg/kg/day ($n = 4$)	30/20 mg/kg/day ($n = 2$)
TC	81 ± 41	59 ± 28	79 ± 35	63 ± 7
GC	37 ± 22	36 ± 19	35 ± 16	37 ± 30
TCDC	18 ± 1	13 ± 4	16 ± 7	23 ± 1
TDC	51 ± 23	54 ± 29	28 ± 9	59 ± 24
GCDC	4.5 ± 2.8	5.4 ± 4.1	4.3 ± 2.0	6.7 ± 3.4
GDC	30 ± 26	42 ± 35	11 ± 6	24 ± 4
TLiC	2.2 ± 1.2	2.2 ± 0.6	1.1 ± 0.4	2.5 ± 1.2
GLiC	1.6 ± 0.9[b]	2.3 ± 2.5	0.9 ± 0.1[c]	1.1 ± 0.2
Total	225 ± 67	214 ± 31	176 ± 34	253 ± 34

Note. Group means ± SD. TC, taurocholic acid; GC, glycocholic acid; TCDC, taurochenodeoxycholic acid; TDC, taurodeoxycholic acid; GCDC, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; TLiC, taurolithocholic acid; GLiC, glycolithocholic acid. Treatment values not significantly different compared to control, using Dunnett's test ($p < 0.05$, 2 tailed).

[a]Includes Monkey 5721 who was sacrificed in moribund condition in Week 20 (Day 137).

[b]$n = 3$.

[c]$n = 2$.

TABLE 5
Liver Tissue PFOA Concentration (μg/g) in Male Cynomolgus Monkeys Dosed Orally with Ammonium Perfluorooctanoate

Monkey ID	PFOA in liver (μg/g)	Time liver tissue was taken
Control group		
5709	0.09	End of dosing, Week 27
5714	<LOQ	End of dosing, Week 27
5715	0.23	End of dosing, Week 27
5725	<LOQ	End of dosing, Week 27
3.0 mg/kg/day dose group		
5702	15.2	End of dosing, Week 27
5706	18.5	End of dosing, Week 27
5717	11.3	End of dosing, Week 27
5721	18.3	Unscheduled sacrifice, Day 137
10 mg/kg/day dose group		
5707	21.9	End of dosing, Week 27
5708	6.29	End of dosing, Week 27
5710	8.86	End of dosing, Week 27
5719	18.8	End of dosing, Week 27
30/20 mg/kg/day		
5704	16.0	End of dosing, Week 27
5713	83.3	End of dosing, Week 27
Control recovery monkeys		
5718	<LOQ	End of recovery, Week 40
5720	<LOQ	End of recovery, Week 40
10 mg/kg/day dose group recovery monkeys		
5712	0.15	End of recovery, Week 40
5716	0.08	End of recovery, Week 40
30/20 mg/kg/day dose group monkeys not dosed through Week 27		
5724	154	Unscheduled sacrifice, Week 5
5711	0.22	Week 27, dosing suspended Week 7
5722	1.41	Week 27, dosing suspended Week 10
5703	1.31	Week 27, dosing suspended Week 12

Note. <LOQ, less than the lower limit of quantitation of 0.019 μg/g.

TABLE 6
Absolute and Relative Liver Weights in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

		Absolute		Relative	
Dose group (*n*)	Body weight	Liver weight	% Control	% Body weight	% Control
0 mg/kg (4)	3947 ± 591	60.2 ± 6.9	100	1.5 ± 0.1	100
3 mg/kg (3)	4486 ± 30	81.8 ± 2.8*	135	1.8 ± 0.1	119
10 mg/kg (4)	4447 ± 498	83.2 ± 9.7*	138	1.9 ± 0.1	122
30/20 mg/kg (2)	3925 ± 583	90.4 ± 4.2*	150	2.4 ± 0.5*	157
Recovery					
0 mg/kg (2)	5410 ± 240	90.2 ± 2.5	100	1.7 ± 0.1	100
10 mg/kg (2)	3932 ± 619	66.0 ± 5.2	73"	1.7 ± 0.1	101"

Note. Table includes only the values from dosed-group animals that received compound through the end of the scheduled dosing period. Values are mean ± SD; weights are given in g. Statistical analysis was not performed on recovery liver weights and relative liver weights.

"Compared to control recovery monkeys as normalized to 100%.

*Statistically significant when compared to control (Dunnett's), $p < 0.01$.

0.01). The two high-dose monkeys that were treated until the end of the dosing period had absolute and relative liver weights that were significantly elevated over the other treatment groups and control. It is noteworthy that the 3 mg/kg/day dose-group monkey sacrificed in moribund condition on Day 137 had a liver-to-body weight percentage of 2.4, which is comparable to the two 30/20 mg/kg/day monkeys dosed through scheduled termination.

All macroscopic and microscopic observations were normal at the terminal sacrifice. This includes key organs such as the liver, adrenal, spleen, pancreas, and testis.

In the 10 mg/kg/day recovery monkeys, absolute and relative organ weights were not increased, nor were there any adverse gross or histopathological findings. This information indicated recovery from hepatic effects observed in this dose group at the end of the dosing period.

Two monkeys were found in moribund condition and sacrificed at unscheduled times during the dosing period. A 30/20 mg/kg/day monkey was sacrificed on Day 29 and found to have edema and inflammation of the esophagus and stomach indicative of dosing injury. This monkey also had liver lesions, including mid-zonal and centrilobular hepatocellular degeneration and necrosis, diffuse hepatocellular vacuolation, and hepatocyte basophilia in centrilobular areas indicative of liver regeneration. Involution of the thymus, a common stress response, and degeneration and necrosis of the heart, probably agonal changes, were also observed in this monkey.

A 3 mg/kg/day monkey was found in moribund condition with hind-limb paralysis and ataxia and sacrificed on Day 137. The hind limbs of this monkey were cool on final medical examination, and ketamine injected into the thigh muscle failed to reach the systemic circulation. This could indicate compromised blood supply to the hind limbs and could also be explained by a neurogenic response without shock, as in an alpha agonist response. The microscopic and macroscopic findings did not reveal evidence of gross spinal cord injury or impaired blood circulation. In addition, the liver of this monkey appeared to be normal; however, as noted previously, the liver-to-body weight percentage was 2.4, comparable to high-dose monkeys dosed through term.

Replicative DNA Synthesis

Cell proliferation in liver, pancreas or testes as measured by proliferating cell nuclear antigen (PCNA) was not different between control and treated groups (Table 7).

DISCUSSION

This experiment was designed to look at the toxicologic effects of APFO in a nonhuman primate model, the cynomolgus monkey, which appears to resemble humans more closely than the rodent in response to peroxisome proliferating compounds. The effects of APFO treatment noted previously in the rat that are thought to be related to the occurrence of hepatocellular, pancreatic acinar cell, and Leydig cell tumors in rats were not observed in the cynomolgus monkeys in this study. Specifically, there was no increase in peroxisome proliferation as measured by palmitoyl CoA oxidase activity. Estradiol was not increased and testosterone was not decreased. No evidence of cholestasis, as evidenced by changes in bile acids, bilirubin, and alkaline phosphatase were observed, and CCK levels did not differ among control and treated groups. The results of this study demonstrated that the liver appears to be the primary target organ in the cynomolgus monkey. Hepatomegaly in the absence of notable histologic findings was present in all dose groups. Decreases in food consumption and body weight were prominent findings in the high-dose group, consistent with effects seen in rats. The exact mechanism of the toxicity of APFO observed in the monkey remains recondite and is the subject of further investigation.

Dose-level selection for this study was based on the results

TABLE 7
Cell Proliferation as Measured by PCNA in Male Cynomolgus Monkeys Dosed Orally with Ammonium Perfluorooctanoate

Monkey ID	Liver (%)	Pancreas (labeling index)	Testes (%)
Control			
5709	0.025	3	22.9
5714	0.075	3	11.0
5715	0.000	3	14.9
5725	0.074	4	27.0
5718[a]	0.025	3	41.5
5720[a]	0.000	3	23.4
3 mg/kg/day			
5702	0.000	4	10.6
5706	0.170	3	21.8
5717	0.099	3	22.8
5721[b]	0.000	NP	13.0
10 mg/kg/day			
5707	0.075	3	15.5
5708	0.050	3	17.1
5710	0.024	3	17.9
5716[a]	0.000	4	10.9
5712[a]	0.073	3	18.8
5719	0.025	3	35.2
30/20 mg/kg/day			
5703[c]	0.075	3	20.2
5704	0.100	3	10.8
5711[d]	0.126	4	21.7
5713	0.075	4	15.8
5722[e]	0.149	3	14.3
5724[f]	ND	3	16.4

Note. Liver, only dark nuclear stained hepatocytes scored as S-phase at 200×. Pancreas, scored subjectively with 4 = islets and exocrine heavily stained (> 50% labeled) and 3 = exocrine stained > islets (100× and 400×). Testes, section farthest from slide label, Leydig cells at 400×. NP, tissue not present on slide. ND, not determined due to too much nonspecific staining.

[a]Recovery animal.

[b]Sacrificed in moribund condition during Week 20.

[c]Dosing suspended Week 12.

[d]Dosing suspended Week 7.

[e]Dosing suspended Week 10.

[f]Sacrificed in moribund condition Day 29.

of a 28-day dose range-finding po capsule study in the cynomolgus monkey. In the dose range-finding study, monkeys were administered the test compound at concentrations of 2 and 20 mg/kg for 28 days. The results of this study revealed no changes in any toxicological or biochemical parameter including organ and body weight measurements. As this was only a 28-day study and the serum half-life of elimination of APFO in the monkey was expected to be greater than 1 month, dose levels of 3, 10, and 30 mg/kg were selected for the 6-month study.

Toxicity occurred early in the study at 30 mg/kg/day, requiring suspension of dosing between Days 12 and 22. After reestablishing dosing on Day 22 at 20 mg/kg, three monkeys again displayed signs similar to those that appeared after dosing at 30 mg/kg. As a result, the quantitative interpretation of responses at the high dose in this study becomes complicated. Two monkeys did tolerate 30/20 mg/kg for the 6-month dosing period. In contrast, 10 mg/kg was not associated with untoward effects in any of the 6 monkeys treated. Therefore, a sharp delineation between effect and no effect for these symptoms occurs between 10 and 20/30 mg/kg/day.

The moribund condition of one 30/20 mg/kg/day dose-group monkey that led to an unscheduled sacrifice reflects the relatively high toxicity observed in the first few weeks of dosing at 30 mg/kg in all high dose monkeys. Symptoms seen in this monkey (aphagia, lethargy, dehydration, and severe loss of body weight) were consistent with those seen in other high-dose monkeys that were experiencing distress after dosing at 30 mg/kg. Therefore, it is likely that the moribund condition of this monkey may have been related to APFO treatment; however, the specific cause remains unclear. Although the esophageal and gastric lesions, the degeneration and necrosis of heart tissue, and the dehydration experienced by this monkey are complicating factors, the contribution of APFO to this monkey's moribund condition cannot be dismissed. Findings in the liver and the concomitant elevations of serum alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase were likely due to APFO exposure.

One could speculate that the extreme elevation in creatinine phosphokinase and the extreme decrease in serum cholesterol along with the necrosis of heart muscle raise the possibility that this monkey may have suffered from rhabdomyolysis and lactic acidosis as a result of reductions in ubiquinone. These findings are consistent with what has been reported for certain HMG CoA reductase inhibitors (statins), especially when given in combination with fibrates (De Pinieux *et al.*, 1996; Omar *et al.*, 2001). Furthermore, one could speculate that the mitochondrial proliferation observed in this study may have been a result of downregulation of HMG CoA reductase by PFOA (Haughom and Spydevold, 1992) leading to a concomitant reduction in ubiquinone leading to decreased oxidative phosphorylation and increased mitochondrial biosynthesis (Berthiaume and Wallace, 2002; Fosslien, 2001). Statins have been shown to decrease ubiquinone and increase the lactate/pyruvate ratio (De Pinieux *et al.*, 1996; Flint *et al.*, 1997).

The *in extremis* condition of the low-dose monkey that was sacrificed on Day 137 is puzzling. This monkey had signs of mild dehydration before initiation of dosing. Normally, this would not be considered a potential confounder; however, one week prior to the unscheduled sacrifice, this monkey exhibited some signs that were seen in the high-dose monkey that was sacrificed early (aphagia, lethargy, dehydration, and severe loss of body weight). In addition, this low-dose monkey had signs of ataxia, restricted hind-limb movement, and an apparent absence of reaction to painful stimuli. No evidence of dosing or cage injury was found. The blood supply to the hind limbs appeared to be severely compromised, although pathological examination did not reveal evidence of spinal cord injury or

impaired circulation. This may suggest a neurogenic response without shock such as alpha agonism. A complete review of the in-life monkey history including review of the clinical and microscopic pathology by a team of pathologists also failed to explain the cause of this monkey's extreme poor health. These findings were nonspecific and, with the exception of low albumin concentration, decreased or no food consumption, and body weight loss, were not consistent with the findings observed for monkeys in the high-dose group that were withdrawn from treatment. It is notable that the liver-to-body weight percentage for this monkey was 2.4, representing a significant increase in liver weight comparable to high-dose monkey values.

Although statistically significant only at the highest dose, there was an indication of increased relative liver weight at all treatment levels. This finding appears to be the most sensitive dose-related effect of APFO observed in this study (Table 6). The increase in liver weights seen following the administration of APFO to cynomolgus monkeys was, at least in part, due to hepatocellular hypertrophy (as demonstrated by decreased hepatic DNA content) which in turn may be explained by mitochondrial proliferation (as demonstrated by increased succinate dehydrogenase activity).

This finding of increased liver weight differs from the findings of a 90-day po gavage study in male and female rhesus monkeys (Griffith and Long, 1980). The rhesus study was conducted with APFO in male and female rhesus monkeys at doses of either 0, 3, 10, 30, or 100 mg/kg/day in 0.5% Methocel. Mortality occurred at doses of 30 and 100 mg/kg. Although an apparent NOAEL of 3 mg/kg was obtained, clinical signs of emesis complicated dosage delivery. A clear determination of a target-organ effect was not obtained in the rhesus study.

A number of special endpoints were selected for inclusion in the study to assist in the determination of potential mechanisms of action. These endpoints were selected primarily because of a suggested link between endpoints observed in long-term rodent bioassays with APFO in which hepatocellular, pancreatic acinar cell, and testicular Leydig cell tumors occurred (Biegel *et al.*, 2001; Pastoor *et al.*, 1987; Riker Pharmaceuticals, 1987).

Changes associated with the potential production of liver tumors in the rat were absent in the cynomolgus monkey. The approximately 2-fold increase in hepatic palmitoyl CoA oxidase activity (a marker for peroxisome proliferation) at the 30/20 mg/kg/day dose (Table 3), is consistent with previous reports for species that are not particularly responsive to peroxisome proliferating compounds (Ashby *et al.*, 1994; Bentley *et al.*, 1993; Cattley *et al.*, 1998). Replicative DNA synthesis, as an indication of cell proliferation, in selected organs (liver, pancreas, and testes) was found to be unaltered in monkeys treated with APFO (Table 7).

Similarly, changes that have been suggested to be associated with the production of pancreatic acinar cell tumors from exposure to peroxisome proliferators in rats include increased serum cholecystokinin concentrations and indications of cholestasis, including alkaline phosphatase, bilirubin, and bile acids (Obourn *et al.*, 1997). These changes did not occur in the cynomolgus monkeys in this study.

In the rat, APFO has been shown to cause sustained increases in estradiol resulting from aromatase induction that have been related to the occurrence of Leydig cell tumors. Among APFO-treated monkeys in this study, no significant changes in levels of the circulating sex hormones, estrone, estradiol, estriol, or testosterone were observed that could be related to treatment.

The data were analyzed both by intergroup comparisons and monkey-by-monkey using each monkey's pretreatment values as the baseline. As expected, a wide range of values was seen both within a particular monkey and among the group of monkeys, which made evaluation of this information very difficult. When looking at the data for trends, with estradiol as an example, values during the treatment period tended to be lower than those seen in the pretreatment and recovery periods regardless of group assignment. Since some of the lower values fell in the 30/20 mg/kg group, it is tempting to consider this as treatment-related, but an objective viewing of this information finds too little cause/effect to attribute the apparent changes to treatment. The failure to see any histopathologic correlation, while not directly bearing on the issue, tends to support the lack of an APFO-related finding.

The results of this study corroborate the relative lack of response of the cynomolgus monkey to the effects of peroxisome proliferating compounds as observed in rats on liver, pancreas, and testes (Cattley *et al.*, 1998; Kurata *et al.*, 1998; Pugh *et al.*, 2000). Assuming that the cynomolgus monkey response to the effects of APFO is more representative of the human than of the rat, the effects observed in rats may have questionable relevance to humans.

There were no clear changes in thyroid hormone homeostasis. All thyroid hormone values were within normal range, and there did not appear to be any relevant histological changes or changes in T_4 or TSH. However, the three high-dose monkeys that were removed from dosing on Days 44, 66, and 81 had T_3 values that trended down compared to pretreatment measurements, and T_3 appeared to trend back toward prestudy values after cessation of dosing. Therefore, the significance of trends in T_3 in these three high-dose monkeys is unclear. Our conclusion is that these apparent changes are best explained by normal variation or stress and are not a direct effect of APFO on thyroid hormone homeostasis.

Although there were only two 10 mg/kg recovery monkeys, there were no test compound-related effects on terminal body weights or on absolute or relative organ weights, indicating that the liver weight increase seen at termination of the dosing period appeared to be reversible. All other macroscopic, microscopic, and clinical observations were normal.

Serum and liver PFOA concentrations did not appear to increase in linear proportion to dose, possibly due to reaching

saturation and steady state in the first several weeks of the dosing period. The reason for the rather high degree of variability in measured serum PFOA concentrations is not completely understood. This variability may be due, in part, to the capsule method of dosing. Also, since PFOA is believed to be eliminated in the bile (Johnson *et al.,* 1984) and reabsorbed, the timing of dosing, blood sample collection, and gall bladder emptying may have played a role. Another factor that must be taken into consideration is the precision of the method, which was ± 30% (interassay, intra-assay, and system). When taken as repeat measures from Week 6 on, the serum concentrations at the two lower dose levels were not statistically different. At the high dose, the concentrations were higher than those at the two lower doses. Similarly, liver PFOA concentrations for monkeys sacrificed at the end of dosing were similar in the 3 and 10 mg/kg dose groups (means of 15.9 and 14.4 μg/g liver, respectively). For the two monkeys in the high-dose group that remained on treatment until sacrifice, the liver concentrations were 16.6 and 86.9 μg/g. The lack of linear proportionality to dose of serum concentrations observed in this study was also observed in an earlier 90-day rhesus monkey study (Griffith and Long, 1980). In that study, the male serum concentrations (n = 1 per dose group) were reported to be 49, 46, and 145 ppm at doses of 3, 10, and 30 mg/kg/day, respectively. In the same rhesus study, liver concentrations were reported to be 3, 9, and 60 ppm at dose levels of 3, 10, and 30 mg/kg/day, respectively. The clearance of PFOA observed in this study suggests that clearance is more rapid than that suggested for retired workers (Ubel *et al.,* 1980). Additional work is in progress to better understand the toxicokinetics of PFOA in human and nonhuman primates.

The doses that were administered in the study were considerably higher than those that are expected from occupational exposure. The American Conference of Governmental Industrial Hygienists (ACGIH) has established a Threshold Limit Value® (TLV®) of 0.01 mg/m^3 as a time-weighted average exposure for workers (ACGIH, 1999). A 3 mg/kg daily dose from inhalation exposure would require an air concentration of 21 mg/m^3, a value considerably above the TLV®.

The authors acknowledge that the small numbers of monkeys used in the study, their heterogenicity, attrition of monkeys due to toxicity and nontreatment-related causes, the need to reduce the high dose early in the study, and the absence of a clear no-effect level may limit the ability to draw conclusions on some points. However, the study was successful in providing insight into the possible mechanism of liver response in the monkey and defining the external and internal dose parameters associated with early toxic response. The study also clearly demonstrates the dramatic demarcation in dose response between a relatively mild response (liver weight increase at the 3 and 10 mg/kg/day dosage) and serious toxicity (dramatic weight loss and one death at the 30/20 mg/kg/day dosage).

In summary, the dose-response characteristics of APFO in the cynomolgus monkey appear to be quite steep. A dose of 10 mg/kg was well-tolerated over a 6-month dosing period with only a minimal effect (liver weight increase); however, 1 week of dosing at 30 mg/kg produced toxicity, and lowering this dose to 20 mg/kg was only tolerated by two of the 6 high-dose monkeys for the 6-month dosing period. A no observable effect level (NOEL) was not determined in this study. The low dose of 3 mg/kg/day is considered a lowest observable effect level (LOEL), based on the suggestion that liver weight may have been increased at 3 mg/kg/day and the uncertainty as to the role of APFO in the moribund condition of a 3 mg/kg dose-group monkey. Effects that have been associated with the development of pancreatic and testicular toxicity in rats were not observed in this study.

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Perfluorooctanesulfonate and Related Fluorinated Hydrocarbons in Marine Mammals, Fishes, and Birds from Coasts of the Baltic and the Mediterranean Seas

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Perfluorooctanesulfonate (PFOS; $C_8F_{17}SO_3^-$), perfluorooctanesulfonamide (FOSA; $C_8F_{17}SO_2NH_2$), perfluorohexanesulfonate (PFHxS; $C_6F_{13}SO_3^-$), and perfluorooctanoate (PFOA; $C_7F_{15}CO_2^-$) were detected in 175 samples of liver and blood of bluefin tuna (*Thunnus thynnus*), swordfish (*Xiphias gladius*), common cormorants (*Phalacrocorax carbo*), bottlenose dolphins (*Tursiops truncatus*), striped dolphins (*Stenella coeruleoalba*), common dolphins (*Delphinus delphi*), fin whales (*Balenoptera physalus*), and long-finned pilot whales (*Globicephala melas*) from the Italian coast of the Mediterranean Sea and in livers of ringed seals (*Phoca hispida*), gray seals (*Halichoerus grypus*), white-tailed sea eagles (*Haliaeetus albicilla*), and Atlantic salmon (*Salmo salar*) from coastal areas of the Baltic Sea. PFOS was detected in all of the wildlife species analyzed. Concentrations of PFOS in blood decreased in order of bottlenose dolphins > bluefin tuna > swordfish. Mean PFOS concentrations (61 ng/g, wet wt) in cormorant livers collected from Sardinia Island in the Mediterranean Sea were less than the concentrations of PFOA (95 ng/g, wet wt). PFOS concentrations in cormorant livers were significantly correlated with those of PFOA. FOSA was found in 14 of 19 livers or blood samples of marine mammals from the Mediterranean Sea. The highest concentration of 878 ng FOSA/g, wet wt, was found in the liver of a common dolphin. Livers of ringed and gray seals from the Bothnian Bay in the Baltic Sea contained PFOS concentrations ranging from 130 to 1100 ng/g, wet wt. No relationships between PFOS concentrations and ages of ringed or gray seals were observed. Concentrations of PFOS in livers of seals were 2.7–5.5-fold greater than those in corresponding blood. A significant positive correlation existed between the PFOS concentrations in liver and blood, which indicates that blood can be used for nonlethal monitoring of PFOS. Trend analysis of PFOS concentrations in livers of white-tailed sea eagles collected from eastern Germany and Poland since 1979 indicated an increase in concentrations during the 1990s. Livers of Atlantic salmons did not contain quantifiable concentrations of any of the fluorochemicals monitored. PFOS is a widespread contaminant in wildlife from the Baltic and the Mediterranean Seas, while FOSA and PFOA were detected only in certain locations indicating their sporadic spatial distribution.

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Introduction

Perfluorinated sulfonates have been commercially produced by an electrochemical fluorination process for over 40 years (*1*). A major fluorochemical produced by this process is perfluorooctane sulfonylfluoride (POSF; $C_8F_{17}SO_2F$). Using this fluorinated compound as a building block, further reactions produce several other fluorinated compounds, including perfluorooctanesulfonate (PFOS) (*1, 2*). These compounds repel water and oil, reduce surface tension, catalyze oligomerization and polymerization, and maintain their properties under extreme conditions. Depending upon the specific functional derivatization or the degree of polymerization, POSF-based compounds may degrade or metabolize to PFOS (*2, 3*). PFOS is stable, chemically inert, and nonreactive and has the potential to bioaccumulate (*4–6*). PFOS has been identified in serum samples from both occupationally and nonoccupationally exposed human populations and in various species of wildlife (*2, 6–11*), and PFOS, FOSA, PFHxS, and PFOA have been detected in human blood (*7*). However, studies describing the occurrence of FOSA, PFHxS, and PFOA in wildlife are scarce (*6, 11, 12*).

The mechanisms and pathways leading to the presence of perfluorinated compounds in wildlife and humans are not well characterized, but it is likely that there are multiple sources of the compound. To understand the spatial distribution of perfluorinated compounds, exposure concentrations were measured in a range of species with different natural histories from various parts of the world. In this study, concentrations of PFOS, FOSA, PFHxS, and PFOA were measured in marine mammals including bottlenose dolphins (*Tursiops truncatus*), striped dolphins (*Stenella coeruleoalba*), common dolphins (*Delphinus delphi*), fin whales (*Balenoptera physalus*), long-finned pilot whales (*Globicephala melas*) and fishes such as northern bluefin tuna (*Thunnus thynnus*), swordfish (*Xiphias gladius*), and in common cormorants (*Phalacrocorax carbo*) collected from Italian coast of the Mediterranean Sea. Furthermore, livers of ringed seals (*Phoca hispida*), gray seals (*Halichoerus grypus*), and Atlantic Salmon (*Salmo salar*) were collected from the Bothnian Bay in the northern part of the Baltic Sea. Similarly, concentrations of target compounds were measured in livers of white-tailed sea eagles (*Haliaeetus albicilla*) collected from inland and coastal regions of eastern Germany and Poland since 1979 until 1999. This provided to an opportunity to evaluate temporal trends in concentrations of fluorochemicals during 20 years. The objectives of this study were to determine the current concentrations of target fluorochemicals in biota from the Mediterranean and the Baltic Seas and to determine their accumulation features.

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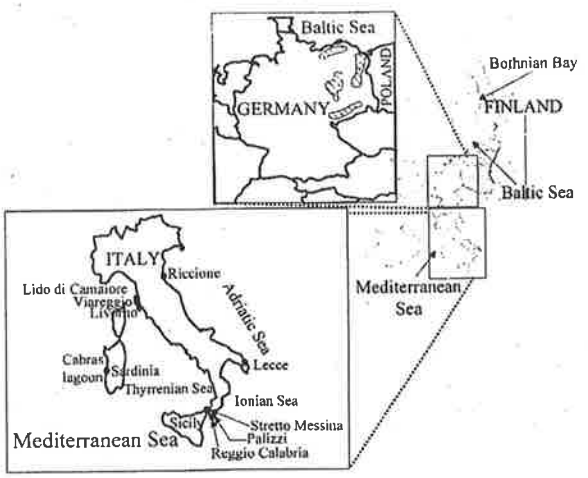

FIGURE 1. Map showing sampling locations (shaded, patterned areas in Germany are eagle sampling sites).

Materials and Methods

Samples. One hundred and seventy five samples of liver and blood of cetaceans (dolphins and whales), fishes, and birds were analyzed in this study. Blood samples of bottlenose dolphins were collected by bleeding (10 mL) captive animals born in delphinariums. In the delphinarium, these dolphins were fed mackerel and herring caught in the Mediterranean Sea and capelin from the North Sea. Livers of common, bottlenose and striped dolphins were collected during mass mortality events along the Italian coast in 1991 (Figure 1). All the dolphins were found stranded, dead along the Adriatic and Thyrrenian Seas. The livers of striped and bottlenose dolphins were freeze-dried prior to analysis. Tissues of long-finned pilot whale and fin whales were taken from animals stranded in the Thyrrhenian Sea. Liver and blood samples of sexually mature (fork length > 110 cm) bluefin tuna were collected in Palizzi, southern coast of Italy. Liver and blood were taken from mature swordfish, which were caught by harpooning in the Ionian – Thyrrenian Seas. Livers of cormorant (*Phalacrocorax carbo*) were collected from the birds that were originally sacrificed in 1997 by the Department of Sanitation-Division of Rearing and Zootechnical Resources due to sanitary regulations. Age class and sex of cormorants were recorded when available. Livers of ringed (*Phoca hispida*) and gray seals (*Halichoerus grypus*) and Atlantic Salmon (*Salmo salar*) collected from the Bothnian Bay in the Baltic Sea were obtained from Finnish Game and Fisheries Research Institute. Salmon were taken from the Rivers Simojoki and Kymijoki in Finland during spawning. All were female salmon, which migrate to the Bothnian Sea, the southern Gulf of Bothnia or the Gulf of Finland. The salmon fast up to 4 months before spawning. Livers of white-tailed sea eagles (*Haliaeetus albicilla*) collected since 1979 until 1999 from eastern Germany and Polish coastal areas of the Baltic Sea were analyzed. Liver samples were wrapped in aluminum foil cleaned with solvents or whirlpac bags and stored frozen at −20 °C until analysis. Sampling locations of fishes, marine mammals, and birds are shown in Figure 1.

Analysis. Concentrations of PFOS, FOSA, PFOA, and PFHxS in liver and blood were measured using high performance liquid chromatography (HPLC) with electrospray tandem mass spectrometry (*7*). Details of preparation of tissues, reagents, and standards have been described earlier (*7*). Analyte separation was performed using a Hewlett-Packard HP1100 liquid chromatograph modified with low dead-volume internal tubing. Ten microliters of extract was injected onto a 50 × 2 mm (5 μm) Keystone Betasil C_{18} column with a 2 mM ammonium acetate/methanol mobile phase starting at 10% methanol at a flow rate of 300 μL/minute, to 100% methanol at 11.5 min before reverting to original conditions at 13 min. For quantitative determination, the HPLC system was interfaced to a Micromass (Beverly, MA) Quattro II atmospheric pressure ionization tandem mass spectrometer operated in the electrospray negative mode. Instrumental parameters were optimized to transmit the $[M-K]^-$ ion for all analytes before fragmentation to 1 or more product ions. When possible, multiple daughter ions were monitored, but quantitation was based on a single product ion. In all cases, the capillary was held between 1.6 and 3.2 kV.

Recoveries of PFOS and other fluorochemicals spiked at the 250 ng level onto sample matrices and carried through the analytical procedure varied (Table 1; Supporting Information). The standards (5 μL of 50 ppm stock) were spiked into liver or blood prior to homogenization. While the recoveries of PFOS spiked to tuna, swordfish, and dolphin livers were 66−140%, the recoveries of PFOS spiked to tuna blood were low (37−47%). The reason for low recoveries of PFOS spiked to tuna blood is not known. Concentrations of PFOS were not corrected for the recoveries of surrogate standard. Similarly, recoveries of FOSA, PFHxS, and PFOA varied depending on the sample matrix. For instance, recoveries of FOSA and PFOA in seal livers were 70 ± 50% and PFHxS was 50%. Recoveries of FOSA, PFHxS, and PFOA in salmon livers were less than 40%, and therefore the data for salmon liver should be considered qualitative. Recoveries of PFOS and PFOA in sea eagle livers were 90 ± 30%, whereas those of FOSA and PFHxS were 54 and 71%, respectively. Based on the duplicate analysis of matrix spike samples, accuracy of the analytical results under the best conditions were ±30% of the reported values. For the estimation of the LOQ, the tissue samples were compared to an unextracted standard calibration curve. For instance, if 5 ng/mL standard is the lowest acceptable standard, and sample had been diluted by a factor of 7, the LOQ is reported as 35 ng/mL. LOQs for fluorochemicals varied from 1 to 72 ng/g, wet wt, depending on the matrix.

Results and Discussion

Mediterranean Sea. Of the four fluorochemicals monitored, PFOS was the most predominant fluorochemical in the tissues analyzed (Tables 2 and 3). PFOS was found in blood of captive bottlenose dolphins at concentrations ranging from 42 to 210 ng/mL (Table 2). The greatest PFOS concentration found in the liver of a common dolphin was 940 ng/g, wet wt (Table 3). Muscle tissue from the same individual contained a PFOS concentration that was 12-fold less than that in liver. Four of five livers of bottlenose dolphins collected from the Adriatic and Thyrrenian Seas contained quantifiable concentrations of PFOS. The mean (±SD) concentration of PFOS in livers of bottlenose dolphins was 54 ± 35 ng/g, wet wt (Table 3). The mean (±SD) concentration of PFOS in livers of striped dolphins was 26 ± 9 ng/g, wet wt. Concentrations of PFOS in livers of bottlenose and striped dolphins were less than those found in cetaceans from the coastal waters of Florida (*8*). Nevertheless, the concentration of PFOS measured in common dolphin liver was similar to those reported for dolphins from the Florida coast. Concentrations of PFOS in muscle and liver of long-finned pilot whales were 52 and 270 ng/g, wet wt, respectively.

Of the other fluorochemicals measured, FOSA was a prominent compound found in livers of dolphins and whales. Concentrations of FOSA in blood of bottlenose dolphins were 1−5-fold greater than those of PFOS (Table 2). Livers of most of the cetaceans (except striped dolphin) contained quantifiable concentrations of FOSA (Table 3). The greatest concentration of 878 ng FOSA/g, wet wt, was found in the liver of a common dolphin. Concentrations of FOSA in bottlenose

TABLE 2. Concentrations of Perfluorinated Organic Compounds in Blood (ng/mL) of Bottlenose Dolphin and Fishes from Italian Coasts[a]

species	sampling area	date of collection	PFOS	FOSA	PFOA	PFHxS	remarks
bottlenose dolphin ($n = 4$)	Riccione (Adriatic Sea)	1997	143 (42–210)	223 (190–270)	3.1 (<2.5–3.8)	4.5 (<1–6.1)	3 males, 1 female; 2–19 years
bluefin tuna ($n = 6$)	Palizzi	Oct-Nov 1999	40 (27–52)	15 (13–19)	<2.5	<1	4 males, 2 ND; 113–158 cm fork length; 25–61 kg
swordfish ($n = 7$)	Stretto Messina (Ionian-Thyrrenian Sea)	July 1999	7.2 (4–14)	15 (1.1–28)	<2.5	<1	3 males, 3 females, 1 ND; 107–190 cm; 15–83 kg

[a] ND = not determined. Values below the quantitation limit were assigned zero when calculating mean.

FIGURE 2. Relationship between PFOS and PFOA concentrations in cormorant livers from Cabras Lagoon, Sardinia, Italy.

dolphins, common dolphins, and long-finned pilot whales were similar to those of PFOS (Table 3). The greatest concentration of FOSA found in the liver of a common dolphin was greater than those found in mink from Illinois (*11*). Occurrence of FOSA in marine mammals from the Mediterranean region indicates the presence of specific sources. PFOA and PFHxS were found in blood of a few individuals of bottlenose dolphins at concentrations ranging from <2.5 to 6.1 ng/mL. PFOA was not found in livers of cetaceans at the quantitation limit of 38–72 ng/g, wet wt. PFHxS was detected in a striped dolphin and swordfish liver at concentrations of 6.8 and 10 ng/g, wet wt, respectively. While PFOS is a metabolic product of several sulfonated perfluorochemicals such as *n*-methyl perfluorooctanesulfonamidoethanol [$C_8F_{17}SO_2N(CH_3)CH_2CH_2OH$] (*13*), FOSA, PFOA, and PFHxS are intermediates in the production of several perfluorinated compounds. FOSA and PFOA are also products used in various applications. FOSA is also a metabolic product of *n*-ethyl perfluorooctanesulfonamide, which is used as an insecticide (Sulfluramid) to control cockroaches, termites, and ants (*14*). PFOA is an impurity in various formulations of perfluorochemicals including aqueous film fire-fighting foams (*5*).

Concentrations of PFOS in cormorant livers collected from Cabras Lagoon in Sardinia ranged from 32 to 150 ng/g, wet wt (mean: 61 ng/g) (Table 3). Mean PFOS concentrations in juvenile birds were not significantly different from those in adults ($p < 0.05$). This is similar for bald eagles collected from the midwestern U.S. (*9*). In general, PFOS concentrations in cormorants were similar to or less than those found in cormorants and other fish-eating water birds collected from the North American Great Lakes (*9*).

PFOA was consistently found in all the livers of cormorants at concentrations ranging from 29 to 450 ng/g, wet wt. Mean concentration of PFOA (95 ng/g, wet wt) in cormorant livers was, on average, 1.7-fold greater than the PFOS concentrations (61 ng/g, wet wt). A juvenile, female cormorant contained the greatest PFOA concentration of 450 ng/g, wet wt, which appeared to qualify as an outlier as this concentration was 4.5 times greater than the standard deviation of the mean. When this outlier value is eliminated, there was a significant correlation between PFOS and PFOA concentrations in cormorant livers (Figure 2). These results indicate that the sources of PFOS and PFOA may be similar for cormorants from Cabras Lagoon in Sardinia. FOSA was found in one of the 12 cormorant livers at a concentration of 89 ng/g, wet wt.

Concentrations of PFOS in blood of bluefin tuna and swordfish ranged from 27 to 52 (mean: 40) and 4 to 21 ng/mL (mean: 10), respectively (Table 2). Similarly, livers of 12 of 13 tuna and swordfish contained quantifiable concentrations of PFOS. The average concentration of PFOS in livers of bluefin tuna (21–87 ng/g; mean: 47) was greater than that determined in swordfish (<1–13 ng/g; mean: 7) (Table 3). The ratios of concentrations of PFOS in liver to blood of

TABLE 3. Concentrations of Perfluorinated Organic Compounds in Livers (ng/g, Wet Wt) of Cormorants, Marine Mammals, and Fishes from the Italian Coast of the Mediterranean Sea

species/tissue	location; date	PFOS	FOSA	PFOA	PFHxS	sample details[b]
cormorant liver	Cabras Lagoon (Sardinian Sea); 1997	43	<38	41	<7	M, adult, 2.4 kg
		57	<38	100	<7	F, adult, 2.2 kg
		49	<38	51	<7	F, adult, 2.3 kg
		98	<38	90	<7	F, adult, 2.33 kg
		34	<38	30	<7	F, adult, 2.7 kg
		150	<38	140	<7	F, adult, 2.2 kg
		91	89	84	<7	F, juvenile, 2 kg
		47	<38	450	<7	F, juvenile, 1.4 kg
		50	<38	47	<7	F, juvenile, 1.96 kg
		43	<38	45	<7	M, juvenile, 2 kg
		32	<38	30	<7	M, juvenile, 2.4 kg
		34	<38	29	<7	M, juvenile, 2.4 kg
bottlenose dolphin liver[a]	Marina di Grosseto (North Thyrrenian Sea); 1991	45	55	<36	<7	
	Livorno (North Thyrrenian Sea); 1991	75	90	<72	<7	240 cm
	Croatia (North Adriatic Sea); 1992	42.5	32.5	<72	<7	M, 288 cm
	Lecce (South Adriatic Sea); 1991	108	32.5	<72	<7	M, 235 cm
	Lido di Camaiore (North Thyrrenian Sea); 1991	<1.4	30	<72	<7	279 cm
	Mar Tirreno; NA	110	139	<38	<19	NA
striped dolphin liver[a]	Lecce (South Adriatic Sea); Aug 1991	40	<38	<72	<7	M, 201 cm
	Viareggio (North Thyrrenian Sea); 1991	22.3	<38	<72	<7	F, 200 cm
	Lecce (South Adriatic Sea); Aug 1991	23.5	<38	<72	<7	F
	Lecce (South Adriatic Sea); Aug 1991	16.3	<38	<72	6.8	F, 201 cm
common dolphin muscle	Giglio Island, North Thyrrenian Sea; Feb 1998	77	142	<38	<19	F, 203 cm
common dolphin liver	Giglio Island, North Thyrrenian Sea; Feb 1998	940	878	<38	<19	F, 203 cm
fin whale muscle	Livorno, coast of Tuscany; Feb 1998	<19	<19	<38	<19	M, 13.8 m, 13.7 tons
long-finned pilot whale muscle	Elba Island, North Thyrrenian Sea; Sep 1996	52	48	<38	<19	160 cm, pup, suckling
long-finned pilot whale liver	Elba Island, North Thyrrenian Sea; Sep 1996	270	50	<38	<19	160 cm, pup, suckling
swordfish liver	Stretto Messina (Ionian-Thyrrenian Sea); Jul 1999	3	<38	<36	10	150 cm, 44 kg
		5	<38	<36	<7	156 cm, 45 kg
		8	<38	<36	<7	140 cm, 53 kg
		<1	<38	<36	<7	161 cm, 61 kg
		13	<38	<36	<7	F, 70 kg
tuna liver	Reggio Calabria (Ionian Sea); Oct-Nov 1999	35	<38	<36	<7	M, 250 cm,
		43	<38	<72	<7	156 cm
	Palizzi; Oct-Nov 1999	87	<38	<36	<7	M, 147 cm, 54 kg
		57	<38	<72	<7	M, 157 cm, 61 kg
		49	<38	<36	<7	M, 158 cm, 51 kg
		21	<38	<36	<7	139 cm
		56	<38	<36	<7	M, 149 cm, 46 kg
		25	<38	<72	<7	152 cm, 56 kg

[a] Values were converted from dry weight basis to wet wt assuming a moisture content of 75%. [b] M = male; F = female. Length of fish and dolphin represents fork length.

TABLE 4. Concentrations of Fluorochemicals in Gray and Ringed Seals and Atlantic Salmon Livers (ng/g, Wet Wt)

species	sex	*n*	age (yrs)	PFOS	FOSA	PFOA	PFHxS
gray seal	male	12	10 (2–25)	243 (148–360)	42[a] (<19–47)	<19	<7.5
	female	15	12.6 (2–33)	190 (140–290)	<38	<19	<7.5
ringed seal	male	10	11.5 (1–21)	490 (130–1100)	<38	34[a] (<19–39)	<38
	female	15	11.5 (4–25)	430 (170–1000)	<38	<19	<38
Atlantic salmon	female	22	Adult	<8	<19	<19	<7.5

[a] Mean of two detectable observations.

bluefin tuna and swordfish were 0.85 and 1.4, respectively. These ratios are 7–12-fold less than those calculated for polar bears from Alaska (*8*). Although the PFOS concentrations in bottlenose dolphins blood were 4–14-fold greater than those in bluefin tuna and swordfish, blood-to-liver ratios of PFOS were less in dolphins than in fishes. This suggests that the distribution of PFOS between liver and blood in fishes is different than in mammals. FOSA was found in all the 13 blood samples of fishes. Concentrations of FOSA (mean: 15 ng/mL) in the blood of bluefin tuna were 2–4-fold less than those of PFOS (40 ng/mL), which was different from that observed in bottlenose dolphins. Mean concentrations of FOSA in swordfish blood was 1.5-fold greater than that of PFOS. Despite the occurrence of FOSA in blood of swordfish and tuna, it was not found in the livers at the quantitation limit of 38 ng/g, wet wt. PFHxS was detected in one of the five livers of swordfish at a concentration of 10 ng/g, wet wt. PFOA was not found at the quantitation limit in any of fish tissue samples.

Baltic Sea. Concentrations of PFOS in livers of gray seals ranged from 140 to 360 ng/g, wet wt (Table 4). Concentrations of PFOS in ringed seals were significantly greater ($p < 0.01$) than those in gray seals. Concentrations of PFOS of up to 1100 ng/g, wet wt, were found in ringed seals from the Bothnian Bay. The greatest concentration was found in a 7-year old male ringed seal, while the second greatest

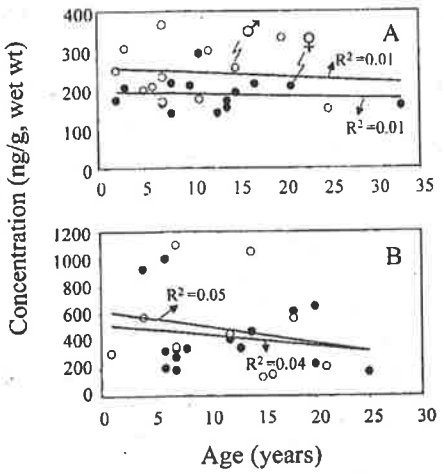

FIGURE 3. Relationship between PFOS concentrations and age in gray (A) and ringed (B) seal livers.

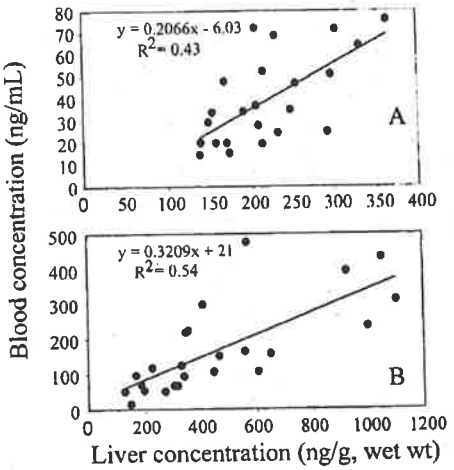

FIGURE 4. Relationship between PFOS concentrations in liver and blood of gray (A) and ringed (B) seals.

concentration was found in a 6-year old female ringed seal. There was no significant difference in the concentration of PFOS between sexes of ringed seals ($p > 0.05$). However, PFOS concentrations in male gray seals were significantly greater than those in females ($p < 0.05$). Earlier studies have reported the lack of significant differences in PFOS concentrations between gender (*8*). The observed differences in the accumulation of PFOS between genders of ringed and gray seals could be due to specific reproductive parameters. Female ringed seals are reproductively mature at about 6 years of age, whereas female gray seals start to reproduce from ages 3 to 5. Earlier parturition may be a reason for lesser PFOS concentrations in female gray seals than in ringed seals. However, several other biological parameters associated with samples such as age may limit the interpretation. Concentrations of PFOS did not increase with age in either ringed or gray seals (Figure 3). This is similar to results observed for mink and marine mammals from the U.S.A. (*8*, *11*). In an earlier study, we reported PFOS concentrations in blood of ringed and gray seals (*8*). The ratios of PFOS concentrations in ringed and gray seal liver and blood were 2.7 and 5.5, respectively. These values were greater than those found in bluefin tuna and swordfish analyzed in this study, suggesting preferential enrichment of PFOS in livers of mammals than in fishes. There was a significant correlation between PFOS concentrations in livers and blood of both ringed and gray seals ($p < 0.05$) (Figure 4). This indicates that blood can be used as a matrix for nonlethal monitoring of PFOS exposures in marine mammals.

FOSA and PFOA were found only in 2 of the 52 samples of seals analyzed. PFHxS was not detected in any of the samples. This was different from that observed in marine mammals from the Mediterranean Sea, where FOSA was relatively prominent in marine mammals. PFHxS was not found in any of the seal livers analyzed.

Livers of 22 Atlantic salmon collected from the northern Baltic Sea were analyzed for target fluorochemicals (Table 4), and none of the salmon livers contained the fluorochemicals at the quantification limit of between 8 and 19 ng/g, wet wt. All Atlantic salmon were adult females and were collected during spawning. The salmon fast for 4 months prior to spawning and the lack of quantifiable concentrations of fluorochemicals in salmon could be attributed to their long fasting periods.

White-tailed sea eagles collected from eastern Germany and Poland contained quantifiable concentrations of PFOS (Table 5), ranging from <3.9 to 127 ng/g, wet wt. These concentrations were severalfold less than those found in bald

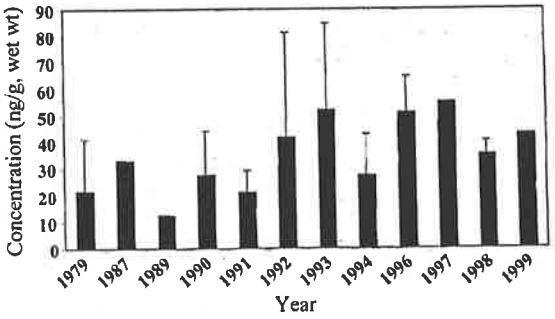

FIGURE 5. Concentrations (mean ± SD) of PFOS in livers of white-tailed sea eagles collected from 1979 to 1999. Sample collected in 1995 was removed from this graph.

eagles from the United States (*9*). Concentrations of PFOS varied widely within the sampling years. However, comparison of concentrations in livers of sea eagles from the 1970s and 1980s (25 ng/g, wet wt) with those from the 1990s (40 ng/g, wet wt) indicated an increase in the 1990s compared to those in the 1970s or 1980s (Figure 5). For instance, mean concentration of PFOS in eagles collected in 1979, 1990, 1992, 1993, 1996, and 1999 were 22, 28, 42, 52, 52, and 45 ng/g, wet wt, respectively. There was a statistically significant increase in the concentration of PFOS in sea eagle livers with time ($p < 0.05$). Sulfonyl-based fluorinated hydrocarbons have been manufactured since the 1950s (*13*). PFOS concentrations in sea eagle livers may be influenced by several factors related to the matrix and accumulation features of fluorinated organics. Livers of birds analyzed in this study were from different ages and sexes. However, no significant correlation existed between PFOS concentrations and age or sex of birds (*9*). Unlike neutral organic pollutants such as PCBs, PFOS covalently binds to proteins in liver and blood plasma (*15*, *16*). Therefore, accumulation of PFOS in higher trophic predators is controlled by a dynamic equilibrium between uptake and elimination or is related protein turnover. On the other hand, neutral lipophilic contaminants such as PCBs tend to accumulate in fatty tissues over a period of time, which results in an age-related increase of concentrations. Therefore, abiotic matrices such as dated sediment or lower trophic organisms may be better indicators for temporal trend analysis of PFOS. FOSA, PFHxS, and PFOA were not detected in livers of white-tailed sea eagles at the quantitation limits of 38, 7, and 40 ng/g, wet wt, respectively. This is different from that observed in the Mediterranean Sea, in which PFOA

TABLE 5. Concentrations of PFOS in Livers of White-Tailed Sea Eagles (ng/g, Wet Wt) from Eastern Germany and Poland[a]

sample	sex	date	wt (g)	location[b]	PFOS	remarks
79/6	F	1979	5700	National Park Muritz	3.9	adult
79/8	F	12/6/1979	4200	Stegelitz	11.3	subadult
79/5	F	17/4/79	6640	Waldlewitz	49.5	immature
87/18	M	4/11/1987	4374	Dassow	40.5	juvenile
M 87/21	F	29/3/87	4325	Dabelow	25.8	adult
89/1	F	24/2/89	6060	Kustrinchen	21	adult
89/6	NA	25/5/89	1445	Rodenskrug	<3.9	juvenile- 8 d old
MV 90/9	F	4/12/1990	3445	Schlicht	35.1	juvenile
90/4	F	12/4/1990	5640	Kotzow	43.2	adult
90/7	M	15/8/90	2970	Melzower Forst	3.9	adult, emaciated
190/3	M	28/3/90	4425	Babke	<3.9	adult
MV91/4	F	1/3/1991	5800	National Park Muritz	8	immature
MV 91/8	M	6/4/1991	4105	Bergfeld	25.6	adult, emaciated
MV 91/10	F	6/5/1991	5517	Julchendorf	20.8	adult
MV 91/12	F	25/6/91	4830	Bossow	30.1	juvenile-70d old
S92/1	F	2/1/1992	4230	Kamenz	38	immature 2yrs
MV 92/21	M	4/11/1992	4315	Axelshof	12.4	immature
MV 92/2	M	5/1/1992	NA	Lahnvitz	23.8	juvenile
MV92/3	F	6/2/1992	3805	Lewitz, Bahlenhusch	127	decomposed, emaciated, juvenile
MV 92/9	M	28/04/92	4280	Near Torgelow	11.9	adult, 6 yrs
BB 92/20	F	28/10/92	5255	Milmersdorf	36.8	adult
BB 93/19	F	1/10/1993	4750	Wilmersdorf	39.8	juvenile
SA 93/17	F	4/9/1993	5420	Stackelitz	23.3	immature
S93/7	F	8/4/1993	5710	Konigswartha	108	subadult
MV 93/2	F	15/2/93	4505	National Park Muritz	37.5	adult, moderate decomposition
MV 94/11	F	10/4/1994	3620	Dobbin/Glave	28.1	adult, emaciated
BB 94/18	M	12/5/1994	3595	Kuhzer lake n. Ruhhof	32.8	adult, emaciated, decomposed
BB 94/2	F	15/1/94	4300	Thomsdorf	10.5	adult
BB 94/4	M	17/2/94	5116	Angermunde-Tantow	16.6	adult
MV 94/19	M	17/5/94	4225	near Karow	58.5	adult
94/6	F	25/2/94	3460	lake at Sproitz	11.5	emaciated, juvenile
BB 94/9	M	26/3/94	4500	Chorin	33.8	immature, moderate decomposition
MV 95/5	F	21/2/95	6500	Kolpinsee/Usedom	120	5 yrs
S96/7	F		3400	Niederspree	65	immature, emaciated
S 97/22	M	28/8/97	3800	Ebersbach-Rodern	99.6	adult
S 98/18	F	28/7/98	4920	klein Krauscha	42.1	immature, moderate decomposition
No3, 28/02/00	M	1996		Western Poland	31.8	immature, emaciated
No4, 2001	F	1996		Western Poland	63.4	adult, liver disease
No5, 3/06/00	M	1996		Poland	44.4	immature, emaciated
No7, 2000	M	1997		Poland	10.7	adult, emaciated
No1, 11/02/00	F	1998		Poland	29.7	adult
No2, 20/01/00	F	1998		Wolsztyn, Poland	33.5	adult, healthy
No6, 2000	F	Oct 2000		Krucza, Poland	62	young
No8, UNK	NA	1999		Poland	24.4	adult

[a] FOSA, PFHxS, and PFOA were not found in any of the sea eagle livers at the quantitation limits of 38, 7, and 40 ng/g, wet wt, respectively. [b] All the locations are in eastern Germany except those indicated as Poland, which are collected from the Baltic coast of Poland.

was relatively prominent in cormorants. This indicates region-specific distribution of these fluorochemicals in biota.

The results of this study suggest widespread occurrence of PFOS in fishes, birds, and marine mammals from the Mediterranean and the Baltic Seas. Concentrations of FOSA in blood of bottlenose dolphins and swordfish from the Mediterranean Sea were greater than those of PFOS. Despite the occurrence of FOSA in blood tissues of fishes, it was not detected in livers. Similarly concentrations of PFOA were greater than those of PFOS in cormorant livers from the Mediterranean Sea. FOSA, PFOA, and PFHxS were found less frequently in seals from the Baltic Sea. Concentrations of PFOS in livers of white-tailed sea eagles collected from eastern Germany and Poland from 1979 to 1999 did not exhibit clear temporal trends. In general, FOSA and PFOA are distributed sporadically in certain species and locations. PFHxS was not found in blood and liver of most of the samples analyzed.

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Supporting Information Available

Recoveries (%) of PFOS spiked at the 250 ng level onto liver and blood matrices (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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